THE PATHOGENESIS OF EXPERIMENTAL SCRAPIE IN INBRED MICE

by

George W. Outram

B.Sc.(Hons.) (Liverpool), B.D.(Hons.) (London).

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The work reported in this thesis has been entirely my own with the exception of technical assistance and some team-project work which is indicated in the appropriate places in the text.

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SUMMARY

An outline is given of the present state of knowledge regarding the pathogenesis of scrapie agents in mice. Emphasis is laid on the unconventional nature of the disease and its causal agent.

A methods section describes the relevant aspects of the special methodology which has been developed in the research institutes where this thesis was written.

An investigation of the incubation periods of three different strains of scrapie agent injected at a range of ages from birth to weaning in three strains of inbred mice shows that the undeveloped state of these hosts at birth causes a considerable modification of pathogenesis: in particular, many newborn mice do not develop scrapie after a dose which would always kill weanlings. Reasons are given for suspecting that the relevant undeveloped organ system is the lymphoreticular system, and that some form of scrapie 'inactivation' must also be involved.

Attempts to throw light on agent pathogenesis by looking for pharmacological treatments which will change incubation periods, produced numerous negative results (outlined) and some positive ones (described in full). Using the i.p. route of agent injection and drug treatment in the weeks immediately before and after infection, significantly changed incubation periods were obtained with prednisone acetate, arachis oil, prednisone acetate + cyclophosphamide, and prednisone acetate + peritoneal-cell provocation with thioglycollate

medium. Preliminary positive results using neonatal treatment with 6-hydroxydopamine and adult treatment with phytohaemagglutinin are also described. Evidence is reported for a prolonging of incubation period of i.c. injected agent by subsequent actinomycin D injections.

Experiments are described in which the peripheral pathogenesis of ME7 scrapie appears to be greatly modified both in terms of incubation period and pattern of lesion distribution in the brain by donor-tissue components. Observations on the histological differences are reported and a number of experiments described which suggest that agent pathogenesis may require <u>specific</u> reactions of an immunological type on the part of the host to donor-specified antigens in the inoculum.

It is shown in a large range of agent/host strain combinations that there are early changes in drinking (and in some cases feeding) habits of mice infected with scrapie. Reasons are given for believing that these are due to an upset of normal brain function by the agent the physiological basis of which is close to the primary lesion due to scrapie, i.e. some derangement of the function of the <u>sinc</u> gene or its immediate product (SECTION 2). The possibility that this is an upset in catecholamine function is discussed.

A number of experiments and observations ancillary to the main sections are collected in five appendices.

Full discussions are given at the end of each SECTION, while in the Final Discussion an attempt is made to bring all the above observations together and to point the way to further research. Several alternative models of scrapie pathogenesis in peripheral organs are briefly reviewed.

ABBREVIATIONS AND SPECIAL TECHNICAL WORDS

i.c.	intracerebral
i.p.	intraperitoneal
s.c.	subcutaneous
CNS	central nervous system
incubation period	the period (in days) between injection with scrapie and the date of confirmed positive clinical scrapie (carefully defined in SECTION 2). This gives end-points which usually have a normal distribution about a mean date shortly before death.
P/C ratio =	$\frac{\text{incubation period after i.p. route (days)}}{\text{incubation period after i.c. route (days)}}$
p/s ratio =	$\frac{\text{incubation period in VM mice (p7p7)}}{\text{incubation period in C57 mice (s7s7)}}$ (same route)
sinc	a gene of unknown function in the normal mouse which governs the incubation period of all scrapie agents so far investigated. (*sinc* - *scrapie incubation*). Two alleles are known: s7 and p7.
lesion profile	a quantitative description of the density and distribution of vacuolar lesions in the *grey-matter* of the mouse brain resulting from scrapie infection.

oedema	a white-matter vacuolation characteristic of certain agent/host strain combinations.
SE	standard error
SD	standard deviation
PA	prednisone acetate
CPA	cyclophosphamide
TG	thioglycollate
6OHDA	6-hydroxydopamine
PHA	phytohaemagglutinin
AMD	actinomycin D
6MP	6-mercaptopurine

SECTION 1

INTRODUCTION TO THE PATHOGENESIS OF SCRAPIE IN MICE

INTRODUCTION TO THE PATHOGENESIS OF SCRAPIE IN MICE

The pathogenesis of scrapie in mice is still very little understood. During the last decade, since the first successful transmission of the disease into this unnatural host, much of the early experimental work was concerned with establishing that murine scrapie was the same as the natural disease in sheep - that it still showed a similar range of unusual biological and physico-chemical properties. The main obstacle to rapid advance has been that the only way of detecting the agent is by injection of living hosts and waiting several months for the disease to develop, while to assay it involves the even longer process of titration in living animals. Since the incubation periods in mice, even with the maximum possible doses are anything from four months to a year and a half (according to the combination of agent strain and host genotype used), and with the LD_{50} end-point doses taking years rather than months to produce clinical signs and death, progress has necessarily been slow.

It is also possible that a full understanding of this disease and the nature of the causal agent will have to wait for extensions of our knowledge in a number of specialised fields, notably, neuro-physiology, the biology of the lymphoreticular system, virology and perhaps molecular biology, so that our relative ignorance is perhaps not surprising. Despite these limitations, the literature on scrapie and other slow-viral encephalopathies is extensive and many reviews have been attempted. The most recent, by Kimberlin (1973) is the most adequate so far, being written by a worker with a wide knowledge of scrapie research.

Scrapie is a natural disease of sheep, and occasionally of goats. Experimentally, however, it has been transmitted to several species of laboratory mammals, mostly rodents. The use of mice as hosts has made possible a much more economical experimental procedure than in sheep or goats, and strict experimental control is only possible by using inbred strains. It is not generally realised, however, that there is little difference in the absolute length of incubation periods in sheep and mice when the same strain of agent is used.

Scrapie is the best known and understood member of a group of mammalian central nervous system diseases, all of which have a similar histopathology and clinical pattern of disease. Various provisional names have been proposed for them, the least objectionable of which is probably 'the slow-viral encephalopathies', although even this assumes a viral origin, which many workers consider unlikely. Another animal disease in the group is transmissible mink encephalopathy (TME) (Marsh et. al., 1969). Two relatively rare human CNS diseases are also included: the well-known affliction of certain tribes in New Guinea called 'kuru' (Hadlow, 1959; Gajdusek et. al., 1966, 1967; Gibbs et. al., 1969) and Creutsfeldt-Jakob disease (Gibbs et. al., 1968). These four encephalopathies are included at a higher level of classification in Sigurdsson's 'slow-viral diseases' (Sigurdsson, 1954) which also contains some instances of true viral infections. In the opinion of some there is sufficient evidence also to add multiple sclerosis to the group (Field, 1970), although there is as yet only indirect and inconclusive microbiological and epidemiological evidence for an infective aetiology, and its pathology is very different

from that of the four slow-viral encephalopathies mentioned above.

Characteristic features of the slow-viral encephalopathies, including scrapie are:

- causal agents of an unconventional kind having a combination of unusual properties which do not occur together in classical viruses;

- long incubation periods lasting from 4 months to 7 years or more, during which the agent appears to replicate slowly but continuously, especially in the lymphoreticular tissues, and eventually in the brain and spinal cord;

- throughout most of this replicative phase there are no obvious signs or symptoms of illness;

- the clinical phase, which occupies only a small fraction of the incubation period, is characterised mainly by various motor upsets, and is probably invariably fatal (it is so, at present, by definition);

- the microscopic lesions are of a degenerative but non-inflammatory nature and are confined to the central nervous system (CNS), although it is possible that all those so far reported are of a secondary nature and have no direct relation to the cause of death.

Experimental scrapie in mice appears to be essentially the same as the natural disease in sheep, and there are no serious reasons for supposing that it is not an adequate model with which to work. There are some differences, however, the most conspicuous being that 'scraping' due to a persistent pruritis is rare in the murine disease (it is not universal in sheep), although it does occur in certain agent/mouse strain combinations (Pattison & Jones, 1968;

Dickinson, pers. comm.). Care is, of course, needed to distinguish this from skin damage due to parasites or fighting. Probably a much more important and interesting difference is the fact that maternal transmission and lateral contagion are found in the natural sheep disease (Dickinson, Young, Stamp & Renwick, 1964, 1965; Dickinson, Young & Renwick, 1966; Dickinson, Mackay & Zlotnik, 1964; Dickinson, 1967) but neither seem to occur in mice (Clarke & Haig, 1971). Reported instances of lateral contagion in mice are likely to be due to oral infection either from oozed inocula or from cannibalism (Dickinson, et. al., 1964; Pattison, 1964). Excretion of infective agent in the urine or faeces during the course of the disease has never been found in sheep or mice.

Work in the laboratories where this thesis was written has demonstrated the existence of many strains of scrapie agent each with its own interactions with different genotypes of mouse host. Consciousness of our incomplete knowledge of this range of variation makes it difficult to be confident about generalisations. With this qualification in mind, the following is an outline of what seems to be the usual course of pathogenesis in those agent/host strain combinations showing the shorter type of incubation period. The reasons for this restriction will become apparent in SECTION 2.

When scrapie inocula are injected by the intracerebral (i.c.) route (as is most frequently done since this gives the shortest incubation periods) most of the agent probably passes immediately into the circulation and within a few days begins to replicate in the organs of the lymphoreticular system. However, there is much evidence to suggest that death after an i.c. injection is caused by the agent which

has remained in the brain and replicated there and not by the considerable amounts of peripherally replicated agent. The main kinds of evidence are:

- the much shorter incubation periods after i.c. than after peripheral injections (e.g. intravenous, i.v. and intraperitoneal, i.p.) and in particular, the failure of i.c. injection of saline to radically shorten the incubation period of i.v. scrapie;
- the brain lesion distribution resulting from intracerebral injection is different from that for peripheral injection and this is not merely due to the cerebral trauma of injection (Fraser, 1971) (lesion profiles - see SECTION 2);
- splenectomy, whether before or after injection, prolongs the incubation period of i.p. but not i.c. injected scrapie;
- hereditarily spleenless mice (Dh/+) show identical i.c. incubation periods to their (+/+) litter mates with spleens, but longer i.p. incubation periods;
- estimates of titre by assay in living hosts show at least a ten-fold higher value by the i.c. than by an i.p. route (Dickinson, pers. comm.; Carp, pers. comm.);
- standard scrapie injections (see SECTION 2) given by the i.c. route to neonatal mice show essentially the same incubation period as weanlings, while the same dose given to neonates by the i.p. route often produces no disease at all (SECTION 3). On the other hand, treatments of the host which have affected

the pathogenesis of peripherally injected scrapie do not affect the incubation period of i.c. injected agent (SECTION 4).

A rise in titre in the brain after an i.c. injection can be detected about 2 months later. The timing appears to be largely under the control of an autosomal gene called *sinc* (an acronym for *sc*rapie *inc*ubation, Dickinson et. al., 1968 and SECTION 2). Also at this time, numerous changes occur in brain physiology (see below) although behavioural changes are not apparent, even to experienced observers without the use of sensitive tests (SECTION 6). Eventually, four or more months after the i.c. injection, clinical signs begin, and within a few weeks the mice are dead.

When, however, the agent is administered by a peripheral route (most commonly intraperitoneal, i.p.) then several extra steps in pathogenesis seem to occur, resulting in both longer incubation periods and lower estimates of titre. Although unpopular with scrapie workers on account of the even longer incubation periods, this route has compensatory advantages of its own and is the one most used in this thesis. It is, for instance, arguably a more 'natural' route than the i.c. one, and there are good reasons for concluding that an i.c. injection bypasses some normal early steps in pathogenesis whereby the host is able to exercise a limited control over the fate of peripherally administered agent. There is the possibility that an understanding of these early steps may hold out hope for discovering methods for controlling the disease. Further, since the replication of the agent in extraneural organs is also under the close control of the *sinc* gene (Dickinson & Fraser, 1969; Dickinson, pers. comm.) the

association is likely to be more accessible to investigation by various types of biological or biochemical interference in the periphery than in the CNS, since the blood-brain-barrier and various other difficulties render the brain relatively inaccessible to biochemical and surgical manipulations.

After a peripheral injection (as after an i.c. one) there is a rapid increase in titre of the agent in the spleen (Eklund, et. al., 1967; Dickinson & Fraser, 1969; Hunter, et. al., 1972). It is probable that this is mainly due to replication there, although it is reasonable to assume that at least some of the increase is the result of accumulation. By 2 months after an i.c. route, and a few days later after i.p. injections the spleen titre seems to plateau (suggesting either site-saturation or a steady turnover of agent either by inactivation or export) (Eklund, et. al., 1965; Dickinson, et. al., 1969). 12-16 weeks after peripheral injections the titre begins to rise in the brain. The mode of transport from the periphery is unknown and there is no satisfactory evidence that it is present in circulating blood. Once in the brain, the peripherally injected agent appears to replicate in the same manner and at the same rate as a centrally injected agent, although there are often considerable differences in lesion profile from the same agent after injection by the two routes (Fraser, 1971 and pers. comm.; Outram, Fraser & Wilson, 1973; and SECTIONS 2 & 5).

It should be emphasised that the above outline is a generalisation from many published and unpublished experiments in these and other laboratories, using various (and often poorly defined)

strains of agent, host genotype and routes of injection. There are agent/host strain combinations that depart radically from this outline (at least in terms of timing) and the exact range of variation is unknown. As a result there is a strong inclination to use operational descriptions and so avoid premature generalisations and this preference results in the frequent use in this thesis of expressions like 'agent/host strain combinations' and 'different combinations'.

The details of the clinical signs also vary considerably with agent and mouse strain; they include: inco-ordination of gait, circling locomotion, swollen bladder and incontinence, priapism, phases of excitibility or somnolence, pruritis, and subtle upsets of behaviour such as changes in drinking and feeding habits (SECTION 6) and reactions in emergence and open field testing.

The known histological lesions are confined to the CNS, but only very general and equivocal correlations have been made between their details and the above range of functional disturbances. The most obvious brain lesions, using simple staining techniques, are the vacuoles in the neurones and the neuropil of the grey-matter, and in some agent/host strain combinations a vacuolation of the white matter (which will be referred to subsequently as 'oedema' to distinguish it from grey-matter vacuolation). These appear soon after the first clearly detectable rise in brain titre, and increase progressively in intensity, apparently in step with this rise in titre (Fraser & Dickinson, 1968; Pattison & Smith, 1963). Other staining techniques show an early hypertrophy of the astroglia with

a slight increase in numbers (Pattison, 1965; Field, 1970; Giorgi, et. al., 1971), fibre degeneration (Fraser, 1969), deposition of neutral fat (Mackenzie & Wilson, 1966) and in some combinations the formation of amyloid plaques (Fraser & Bruce, 1973). Several biochemical changes have also been described, including changes in the metabolism of DNA, histones, ATP, polyamines, glycosidases, and fucose. These are reviewed by Kimberlin (1973) but their exact significance is still uncertain and their status as primary or secondary effects in doubt (Little & Adams, 1971).

The pattern of the distribution in the brain of the vacuolar lesions in the grey-matter of mice has been subjected to much analysis in these laboratories (Fraser & Dickinson, 1973 and SECTION 2). There is considerable variation between different agent/host strain combinations coupled with a remarkably high degree of consistency within them.

Despite the discovery of all these scrapie-associated phenomena and of the detailed analysis of some of them, the cause of death is unknown and there are as yet no methods of prophylaxis or cure once infection has become established (but see SECTION 4).

The necessity for special approaches in scrapie research

Much of the work so far on scrapie has drawn attention to the unconventional nature of the disease and its causal agent, which has tended therefore to be merely negatively characterised. This state of affairs provides a great challenge and requires a readiness to work in ways that are untrammeled by the usual notions of how to

investigate viral diseases. Scrapie has proved refractory to a wide range of conventional approaches, which, although they had to be tried in the normal course of events, have had to be abandoned or drastically modified. For instance, while it is usual to bemoan the long incubation periods and to endeavour therefore by all means to reduce them, this is compensated by the fact that operationally exact experimental replicates give extraordinarily similar results despite the long times involved. This fact is itself of great biological interest, demanding an explanation, but it also permits an economic style of experimentation using numerous small-scale pilot experiments for the initial exploration of interesting observations.

The necessity for a special approach to this disease is best demonstrated by summarising the outstanding anomalous features as compared with conventional viruses.

1. Very long incubation periods but of very consistent length and showing a standard error of the mean of only a day or two (especially after intracerebral injections). The length of the incubation period also increases in a highly predictable way as the dose is decreased. The proportionate increase of incubation period for the i.p. route, compared with the i.c. route, is relatively constant for each agent/host strain combination (Dickinson & Outram, 1973; Dickinson, pers. comm.).

2. No conventional immunological responses by infected hosts have been demonstrated (Chandler, 1959; Gardiner, 1966; Haig & Clarke, 1965; Clarke & Haig, 1966; Gibbs, Gadjusek & Morris, 1965; Clarke, 1968; Katz & Koprowski, 1968) nor is there any sign of a

general depression of immune responsiveness as is known to occur with some viruses (Clarke, 1968; Gardiner & Marucci, 1969). Because of the association of the agent with the lymphoreticular system, numerous experiments have been published attempting to change incubation periods by using immunosuppressive techniques. For instance, treatment with cyclophosphamide (Worthington & Clarke, 1971) or adult thymectomy followed by irradiation and reconstitution with foetal liver (McFarlin, et. al., 1971). Other investigators have tried neonatal thymectomy (Gibbons & Hunter, 1967) and antilymphocytic serum (Hirsch, 1970) but unfortunately chose to inject scrapie by the i.c. route. All such treatments have failed to influence the course of the disease. This lack of immunological responsiveness to scrapie is also serious from an experimental point of view because it excludes the use of techniques like serological identification, visualisation by immunofluorescence, and estimation by immunoassay. Recently, Field et. al. (1972) have reported some subtle changes in lymphocytes taken from scrapie animals which may have an immunological basis, but it is too soon to assess the significance of these findings.

3. There has been no success with attempts to use tissue-cultures as assay systems, or for rapidly preparing high titres of relatively pure agent. A slow rate of replication in cultures of cells of unknown type derived from infected mouse brains has been demonstrated (Clarke & Haig, 1970) but it has not been possible to show infection of normal tissue cultures with agent. The infected cultures of Clarke and Haig have not been usable for biochemical investigations because of the lack of any acceptable kind of control cells, nor do

they release active agent into the supernate.

4. Progress in understanding the agent in conventional terms has been hampered by an inability to purify it or even to produce any concentration of activity higher than that found in crude brain homogenates. There is evidence, at least in the case of some agents, that infectivity is most strongly associated with extracted plasma-membrane fragments. This does not necessarily tell us anything about the in vivo site of activity, and interpretation of such findings is difficult. This has not deterred some from attempting to construct models of the scrapie agent in terms of modified host-membranes (Gibbons & Hunter, 1967; Adams, 1970). These are almost certainly premature and even now cannot account for many important findings, notably those involving the interactions of agents with the sinc gene (see below and SECTION 2).

5. The agent has never been identified with the electron microscope, although numerous peculiar features of infected cells have been reported (Chandler, 1967; Field & Narang, 1972). It is likely that this is because the agent is very small, associated with host tissue, perhaps only present as a few units per cell, and may be visually indistinguishable from many normal constituents of cells. Estimates of agent particle size using filtration and irradiation techniques (both subject to numerous difficulties in interpretation especially when crude tissue suspensions have to be used) have given measurements of 20-50 nm. in the smallest physical dimension (Kimberlin et. al., 1971) and an inactivation target size of 7 nm. (Alper, et. al., 1966).

Estimates of the average numbers of infectious units in tissues are in the order of 1 unit per brain cell (in terminally affected animals) and 1/50 for the spleen. It is not known if this represents a high infection of a few cells or a low infection of many. It is not difficult to see how these kinds of numbers and dimensions could make difficulties for electronmicroscopists.

6. There are practical problems in estimating the titre of scrapie agents in infected tissues. The only fully satisfactory method at present is by titration in whole hosts to find the LD_{50} dose. It should be emphasised, however, that this is only an operational definition of titre and the estimates are known to vary according to the precise test conditions including the host genotype (Dickinson & Meikle, 1971). While it is true that when a particular agent/host-strain combination is well understood, titre can be estimated from the incubation period of a single dilution, it is necessary to be very careful since a change in incubation period can occur from other causes even when the titre remains the same.

7. The existence of numerous strains of agent with a wide variety of biological properties raises other potential problems. For many types of experiment it is important to be certain that one is using only a single agent and not a mixture of strains. The evidence is that affected sheep and goats are often infected with more than one strain of agent: this conclusion is based upon much work in these laboratories involving transmission to mice and the analysis of subsequent passages in mice of different genotypes (Fraser & Dickinson, 1973). Since these agents can differ <u>considerably</u> in terms of their incubation

periods, pathology, and resistance to physical and chemical treatments, the use of mixtures (such as the Chandler 'agent') in experiments could lead to serious errors of interpretation if the presence of more than one agent was not recognised. The above mentioned scrapie material has been very widely used in this country and abroad, and this is one of a number of reasons for having serious misgivings about some of the generalisations regarding the properties of "scrapie" that have been based upon it.

8. The genotype of the host can also have a marked effect upon the incubation period and patterns of lesion distribution in the brain caused by a particular strain of agent. One gene is known in mice that can more than double or halve the incubation periods of specified agents according to the allele or combination of alleles present in the host (see SECTION 2). Clearly, if one is using experimental mice that are segregating for this gene it will provide a source of great variation in incubation period within experiments and a cause of uncontrolled variations between replicates - unless one uses prohibitively large numbers of animals.

9. Most scrapie agents show a remarkable degree of resistance to inactivation by standard sterilizing procedures, including conventional autoclaving, irradiation treatments, boiling, and immersion in formalin, although variation between strains is still largely unexplored and seems to be greater than is generally appreciated. This puts a question mark against some attempts to speculate about the nature of 'the scrapie agent' on the basis of this resistance. It is also important to recognise that without stringent precautions, these properties

constitute a danger, perhaps to personnel, but certainly to experimental results, because of the greatly increased possibility of contamination in general, and cross-contamination (of strains) in particular.

10. Finally, and largely in consequence of the previous nine features of scrapie, there is as yet no widely accepted model for the scrapie phenomenon, nor an agreed methodology. Instead one finds groups of workers in separate centres pursuing their own lines of research in relative isolation from each other and with only enough common ground for misunderstanding. This is a situation common in new scientific fields, which leads inevitably to difficulties in understanding, assessing and using the results of others - and sometimes to controversy.

Tactical considerations in consequence of the foregoing

The peculiar nature of scrapie as thus outlined obviously requires special tactics for its investigation. This thesis work is modelled on the methods which have been developed by A.G. Dickinson and his colleagues in Edinburgh (see SECTION 2). The chief tactical procedures of this method are:

1. The avoidance of premature specialisation by advancing on a broad biological front, endeavouring always to identify primary effects of the agent which may eventually merit more specialised attention.

2. A concentration on the study of biological variation since this will help to identify primary effects which will tend to have a wide generality from secondary ones which may not. This approach has already identified a gene (sinc) whose two known alleles (s7 and p7)

control the onset of replication of all agents so far tested and in such a manner as to suggest a very close relation between gene function and agent replication (Dickinson & Meikle, 1971).

3. To be constantly on the look-out for new scrapie-associated phenomena in experiments designed for other purposes. This requires a close involvement of the research staff with the experimental animals throughout the incubation period.

4. To open up investigations of interesting new findings with a range of small-scale pilot experiments that rely to some extent upon previous experience with the particular agent/host strain combination to provide controls. Larger experiments with more and tighter controls are not designed until these pilots have shown the scope of the phenomenon. Investigation by titration, which is often necessary for the purposes of clarification is not usually begun until several years after the initial observation.

SECTION 2

METHODS: AN OUTLINE OF THE RELEVANT METHODOLOGY OF THE JOINT SCRAPIE UNIT OF THE ANIMAL BREEDING RESEARCH ORGANISATION AND THE MOREDUN RESEARCH INSTITUTE, EDINBURGH.

2.1 GENERAL INTRODUCTION TO THE SCRAPIE METHODOLOGY DEVISED IN THE A.B.R.O. - MOREDUN INSTITUTE SCRAPIE UNIT.

On account of the special features which are associated with scrapie research as outlined in SECTION 1, it has been necessary to devise appropriate methods for investigating this disease. A strategy has been developed by Dr. A.G. Dickinson and his colleagues in the above unit which uses certain conventional virological methods supplemented by several original procedures. Since this composite approach forms the foundation of the methods used in this thesis, it is necessary to give some account of it by way of introduction and to provide an overall 'Materials and Methods' SECTION to obviate the need for repetition later.

The most characteristic feature of this approach to scrapie research is an emphasis on the need to analyse the full extent of biological variation in the disease. In this way it avoids the dangers of the more obvious procedure which is to use only the quickest, most convenient host/agent combination in attempting to investigate either the agent itself by direct physico-chemical techniques or in the study of pathogenesis. The point is that there are so many secondary features of the disease, many of which are only associated with certain specific agent/host strain combinations, that it is extremely easy to waste resources pursuing such features without any hope of revealing new fundamental knowledge about the disease and its causal agent. Without discounting the importance and possibility of occasional lucky breaks, the chances are that the best way to identify fundamental features is by a broad biological approach in which the generality of

specific observations can be checked. Only when such primary aspects have been identified with certainty will it be time for specialist investigations. This procedure is analogous to that of genetics before the elucidation of the molecular nature of the gene and its mode of operation. Not only was this classical work extremely fruitful in making relevant discoveries, despite the ignorance about basic mechanisms, it provided the foundation on which molecular biology was eventually to be built. Moreover, even now the classical procedures are by no means redundant since a large amount of clarification remains to be done and molecular biology has not supplied all the answers which some expected of it. It is argued, similarly, that in scrapie research at the present time, the most appropriate methodology is a patient analysis of its general biology, in particular of the kinds of variation that are displayed and their interrelations, without being greatly worried about the lack of a model of the central processes. Attempts to construct such models are hardly likely to be correct at this time since they can only be based upon fragmentary knowledge (what chance would even the most knowledgeable biologist have had of providing an adequate model of the gene in say 1930?).

The outstanding achievements of the general biological approach to scrapie in mice used by this unit in the past decade have been, firstly, to develop several refined parameters of measurement based on two types of observation, namely, the incubation period and the lesion profile in the brain, and secondly, to show that a number of natural and experimental variables have a profound effect upon the pathogenesis of scrapie in mice as shown by changes in these

parameters. The chief sources of scrapie variation that have so far been identified are:

- the strain of the agent, of which 8 different ones are known in some detail and about 10 others more tentatively (some of the best characterised strains have been re-isolated several times; Dickinson & Meikle, 1971; Fraser & Dickinson, 1973);

- some form of interference between different strains of agent in mixed infections producing prolonged incubation periods and interpreted as competition for replication sites (Dickinson, Fraser, Meikle & Outram, 1972);

- the genotype of the host, especially with respect to the gene sinc of which two alleles s7 and p7 exercise a very profound influence upon the incubation period of all strains of agent so far tested (Dickinson & Meikle, 1971; Dickinson, pers. comm.);

- operational procedures such as dose of the agent, route of injection, the infectious tissue used, and the strain of the donor (Dickinson & Meikle, 1969; Fraser & Dickinson, 1973; Dickinson & Outram, 1973; Outram, Fraser & Wilson, 1973);

- integrity and maturity of the lymphoreticular system where peripheral routes of injection are involved (Fraser & Dickinson, 1970; Dickinson & Fraser, 1972; Outram, Dickinson & Fraser, 1973).

2.2 OUTLINE OF METHODS

The main procedures that have achieved these results are:

1. The use of several standard agents which have been obtained, at least in partially purified form by multiple passage in specific strains of mice, with cloning techniques in some cases.

2. The use as hosts of numerous, fully inbred strains of mice and genetically controlled crosses, housed in a uniform environment.

3. The use of very rigorous aseptic procedures for the removal and storage of tissues and the preparation and injection of infectious inocula which are designed to prevent contamination and cross-contamination. These procedures, in conjunction with those described in 1 and 2 above, give results which are so consistent that much smaller numbers of animals can be used than would otherwise be necessary to obtain accurate results. The degree of control which has now been achieved permits valid comparisons to be made with a corpus of reference standards which minimise the number of types of contemporary controls needed in pilot experiments.

4. The scoring of incubation periods and the topographical distribution and density of brain lesions is done using coded mouse- and tissue-samples, in which the scorer is unaware of the specific nature of the experiment and of any detailed expectations regarding the results. Also, appropriate degrees of randomisation of individual mouse treatments <u>within</u> cages are used, so that group-behavioural interactions among cage-mates are not confounded with treatment groups.

5. The very full recording of all biological and clinical details of every individual mouse from birth to post-mortem analysis, including those of an incidental nature whose significance may only become apparent much later.

2.3 MATERIALS AND METHODS (GIVING DETAILS RELEVANT TO THIS THESIS)

The following paragraphs will expand the above points where they are especially relevant as a background to SECTIONS 3-6.

2.3a Inbred Mouse Strains. More than 20 inbred mouse strains have been used in these laboratories. Table 2.1 shows some of the incubation period characteristics of the agents and mouse strains which have been used in this thesis. All the incubation periods quoted are for an intracerebral (i.c.) injection of lightly centrifuged (500g, 10 mins.) 1% saline homogenate of terminally affected mouse brain.

These data illustrate the wide variety of lengths of incubation that are obtained with different agent/host strain combinations, and the remarkably small variation about the group mean which obtains even with the longest of them. Of particular importance are the effects of the various *sinc* genotypes. The effects of this gene are almost without precedent in genetics (although systems with some similarities are known in yeast, Zimmermann & Gundelach, 1969) and the only simple method of understanding the results which its two alleles produce, is in terms of a very close relation between one of the immediate products of the gene (or the gene itself) and the requirements for scrapie replication. The incubation periods of an agent in the two

Table 2.1

Mouse Strain	Abbrev-iation used	sinc genotype	Characteristic i.c. incubation periods * Agents				
			ME7 group				22A group
			ME7	79A	22C	80A	22A
C57BL/FaBtDk †	C57	s7s7	167 ± 1	150 ± 2	178 ± 1	180 ± 2	460 ± 3
BALB/cLacDkf	BALB	s7s7	178 ± 1	163 ± 1	189 ± 1	196 ± 2	460 ± 4
BRVR/SrDkf	BRVR	s7s7	169 ± 2	146 ± 2	-	182 ± 2	480 ± 5
A2G/LacDkf	A2G	s7s7	180 ± 3	163 ± 1	-	-	475 ± 7
RIII/FaDk.rorо	RIII	s7s7	156 ± 2	138 ± 1	197 ± 2	172 ± 2	408 ± 8
VL/Dk	VL	s7s7	170 ± 2	154 ± 1	-	181 ± 0	-
VM/Dk	VM	p7p7	324 ± 3	295 ± 1	448 ± 4	417 ± 6	195 ± 2
(C57BLxVM)F_1	F_1	s7p7	240 ± 2	255 ± 2	261 ± 4	257 ± 4	571 ± 7

† Designations of the committee on Standard Genetic Nomenclature (Staats, 1964, 1966).

* in this and all subsequent tables incubation periods are given in days ± SE.

homozygotes p7p7 and s7s7 constitute the basis for the grouping of scrapie agents into two classes. The 'ME7 group' of agents shows the shorter incubation period in C57 mice (s7s7; s7 = short incubation with ME7) while they have a much longer incubation period in VM mice (p7p7; p7 = prolonged incubation with ME7). On the other hand, the '22A agent group' shows a short incubation period in VM mice and a prolonged incubation period in C57 mice. Other members of this 22A group are known but were not used in this thesis.

The incubation period in the F_1 (s7p7), depending on the particular agent, is either intermediate between the two homozygotes, or as long as the longer one, or even significantly longer than either homozygote (termed overdominance). (See Table 2.1 and Dickinson & Meikle, 1971). This interaction between scrapie agents and some immediate product of the sinc gene in order for the agent to replicate, is the most primary effect yet discovered in scrapie pathogenesis, and it would not have been detected without the discovery of variation at the sinc locus. However, the role of this gene in the uninfected mouse is not yet known and there are no valid grounds for speculating about its normal function.

2.3b Scrapie Agents. At least 18 different scrapie agents have now been isolated in this Unit, but they vary in current passage state and consequently in the reliability of detailed information about their characteristics. The agents are distinguished by operational parameters such as the lesion profile and their relative incubation periods in different genotypes of mouse - although the molecular bases of their differences are not yet known.

All agents have a passage history that leads back eventually to diseased sheep or goats, although the details are sometimes complex, and in the case of 3 agents open to alternative interpretations regarding the true source. Table 2.2 shows the origins and passage histories of the agents used in this thesis. One of the 5 shown, ME7, is the most stable known and its properties are unaffected whether it is passaged repeatedly in either C57 or VM mice. It is not yet known whether this holds true for the other 4 agents.

2.3c Uniform Host Environment. The use of inbred mice provides for the control of the internal environment of the host and it is wise on general principles for quantitative measurement in biological systems, to exercise as much control as possible over the external environment of the host also. Accordingly the rooms, which house about 8000 mice, are maintained by air-conditioning at a temperature of 21° C and a R.H. of 45-55%. Although indirect sunlight illuminates the rooms, seasonal variation is partially counteracted by electric lighting for 12 hrs. every day throughout the year. The animals are caged, up to six at a time, in polypropylene cages with welded wire, stainless-steel lids, shaped to hold an overhead supply of dry pelleted food (Spiller's steam-heated diet) and a rigid plastic drinking bottle with a glass drinking tube. Food and water are supplied ad libitum. Once a week, immediately after scoring of clinical signs, the mice are transferred to clean cages containing sterile litter. Fresh drinking water is given at least twice a week. All cages are checked daily. The normal procedure and precautions for the maintenance of a SPF colony are observed, and

Table 2.2 Sources of Scrapie Agents

Agent Designation	Source and history prior to passage to inbred mice
ME7	2nd passage brain from random-bred Moredun-stock mouse. Originated from the spleen of a Suffolk sheep naturally affected with scrapie (Zlotnik & Rennie, 1963).
79A *	Brain pool from goat with 'drowsy' form of experimental scrapie (Pattison & Millson, 1961) at the 8th goat passage; it is possible that this agent does not originate from scrapie in sheep (Dickinson, pers. comm.)
80A †	Brain pool from goat with 'scratching' form of experimental scrapie (Pattison & Millson, 1961) at the 5th goat passage.
22C †	Cheviot sheep brain pool at the 21st sheep passage of SSBP/1.
22A †	Cheviot sheep brain pool at the 21st sheep passage of SSBP/1.

† These agents stem from a common origin at the 16th sheep to sheep passage of Cheviot sheep scrapie brain pool of the Moredun Institute (SSBP/1). Prior to its isolation in goats, this was further passaged twice in sheep at Compton (firstly in Cheviots, and then in the Welsh Mountain breed). The isolation in mice of the source which yielded 22A and 22C was passaged a further 5 times through Cheviot sheep at the Moredun Institute.

80A and 22C are probably the same agent: they both stem from the SSBP/1 pool and have similar properties even though their lineages differ by 7 sheep and 5 goat passages.

22A is standardly passaged in VM mice (sinc p7p7): the other agents standardly in C57 mice (sinc s7s7).

* 79A is probably the chief constituent agent of the widely used "Chandler-strain" of scrapie.

access is strictly limited.

2.3d The Preparation of Inocula and Injection Procedures. The mice are infected with scrapie by injection with inocula derived from crude tissue homogenates in saline: these inocula are the supernatant fluids of centrifuged homogenates and are referred to as "supernates" for brevity. The homogenates may be of sheep or goat brains or spleens, but more usually they are from mouse tissues. Some sources for this thesis were homogenised pools of several brains, while others were single half-brains or spleens that had not been homogenised. Tissues were stored at -30° C in labelled, screw-top glass bottles. The reason why only half the brain is usually frozen is that it is normal to send the other half for histological examination. The infectivity of the frozen tissues survives unimpaired for many years at least.

Supernates were prepared as follows. The tissue was thawed and a portion removed and weighed using aseptic techniques. It was homogenised in sterile isotonic saline (usually to give a 1% or 10% w/v homogenate) using a high-speed, teflon-in-glass, shearing homogeniser and giving about 20 strokes of the plunger. The homogenate was then lightly centrifuged at either 500g for 10 mins., or 2000g for 15 mins. These alternatives relate to previous standards which had been used and were adopted in this work for confirmity. Very occasionally an unspun homogenate was used as when it was required merely to detect the presence of agent even at low titre. For the convenience of small experiments using only a few animals, some aliqots of supernates were frozen at -30° C. When required for use these

were thawed, agitated in the syringe and injected as usual. A titre check on one such 79A frozen supernate showed an estimated loss of titre of 1 log LD_{50} units after 750 days of storage.

Injections were nearly always by the intraperitoneal (i.p.) route although the i.c. and the oral routes were occasionally used. All scrapie injections in this thesis used a volume of 0.02 ml., without anaesthetic and employing a 26 G disposable hypodermic needle and syringe. The above details will be referred to in the thesis by the expressions "standard supernate" and "standard frozen supernate". The former expression means 0.02 ml. of a supernate from homogenate centrifuged at 500g for 10 minutes, and the latter refers to the same supernate after it has been frozen and then thawed at a later date. When preparative procedures depart from these standards the details will be given.

The mice to be injected were held on a sterilized stainless-steel platform. This, and other metal stands, instruments and homogenisers were sterilized after each usage by four, one hour cycles at 26 p.s.i. (130° C) in a gravity displacement autoclave. Other glass-ware and syringes which had been used for infected tissues were disposed of by incineration.

2.3e Incubation Periods and Related Parameters. Since scrapie agents cannot be located, measured or characterised by direct observation and other straightforward techniques, it is necessary to use the clinical responses of the host as the main indicator of the progress of the disease. For the i.c. route the dose/incubation period response curve for each agent is known very precisely, and as

a result it is often possible to estimate the titre of agent in a single dilution of a particular homogenate with a high degree of accuracy using the mean incubation period in about 10 mice. Only in special cases, therefore, is it necessary to conduct a full log-dilution titration which requires at least 400 days, and about 50 mice.

"Incubation period" can be loosely understood in various ways, but in these laboratories it is measured using rigidly defined criteria (Dickinson, Meikle & Fraser, 1968). Injected mice are scored once a week during the afternoon on a day in which no husbandry routines have previously been carried out. Each mouse, individually identified by cage number and ear notches, is classified as 'unaffected', 'possibly affected' or 'definitely affected' with certain specified clinical signs of scrapie and the scoring is done 'blind' - that is without knowledge of how each individual mouse has been treated. Scoring is performed by trained assistants, whose day to day familiarity with the particular strain of mouse in both health and disease, especially fits them for making these assessments. The end-points for calculating the incubation period are defined as the day on which an animal receives a third consecutive weekly score of 'definitely affected' or the day on which a fourth such score is given within five consecutive weeks, or the day on which it is killed in extremis with scrapie, if less than two weeks after its first definite scrapie score, or it is found dead having received a 'definite scrapie' score during the previous 7 days. This is a buffered system giving results that show a normal distribution about the group mean, (except at limiting dilutions when there is skewing),

which permits the valid use of conventional statistical techniques.

There are some other important parameters which are derived from the incubation period data. They are, the P/C ratio, the p/s ratio and the degree and direction of the dominance of the alleles of sinc with respect to each agent's incubation period.

$$\text{P/C ratio} = \frac{\text{incubation period after i.p. route (days)}}{\text{incubation period after i.c. route (days)}}$$

Values for this ratio range from 1.2 - 2.0 for a 1% homogenate (Dickinson & Outram, 1973 and Dickinson, pers. comm).

$$\text{p/s ratio} = \frac{\text{incubation period in VM mice (p7p7) (days)}}{\text{incubation period in C57 mice (s7s7) (days)}}$$ using same route. Values for this ratio range from 0.42 - 2.56 for the i.c. route (Dickinson, 1970; Dickinson & Meikle, 1971).

The differences in degree and direction of dominance of the alleles of sinc are illustrated by the incubation period values in Table 2.1.

2.3f Lesion Profile. This is the second important parameter developed in these laboratories for measuring host response to scrapie infections (Fraser & Dickinson, 1968; Fraser & Dickinson, 1973). It is a method of measuring and expressing the distribution and intensity of grey-matter vacuolar lesions as they occur in nine selected regions of the mouse brain. It provides an objective, overall description of lesion distribution, which can be shown to depend upon a variety of factors. In every instance that has been fully analysed (which now amounts to 5 agents) any agent that can be distinguished using the incubation period parameters, can also be

distinguished by its lesion profile. Thus it provides an independent method of checking agent identification. This lesion profile, unlike the absolute incubation period, is not affected by the dilution of the inoculum: with the ME7 agent this finding has been established for doses over 6 orders of magnitude. Although it is unaffected by the precise site of injection in the brain (Fraser, 1971), the lesion profile for a particular agent can be significantly changed by a number of other factors. These include the strain of the host, the strain of the donor, the infected tissue used (spleen or brain) and the route of injection (Fraser & Dickinson, 1970; Fraser, 1971). The fundamental bases for these differences in lesion profiles, such as that of the relation between lesions and the distribution of agent or cause of death are still unknown.

The histological scoring procedures are as follows: five transverse paraffin sections, 7 microns thick, prepared from each brain by the method of Mould, Dawson, Slater & Zlotnik (1967) are stained with haematoxylin and eosin. Nine regions of grey-matter are examined for degree of vacuolation, namely (1) the dorsal medulla; (2) the folia of the cerebellar cortex (nodulus); (3) the superior colliculi; (4) the hypothalamus (generally); (5) the thalamus (central); (6) hippocampus; (7) the paraterminal body (septum); (8) the posterior cerebral cortex in the region dorsal to the corpus callosum; and (9) the anterior cerebral cortex at the level of the septal nuclei. Each area is given a score from 0-5 depending on the degree of vacuolation which is present. The five degrees of brain damage may be verbalised as follows: (1) a few vacuoles, widely and unevenly scattered; (2) a few vacuoles, evenly scattered; (3) moderate

number of vacuoles, evenly scattered; (4) many vacuoles with some confluence and (5) dense vacuolation with most of the field confluent. A useful approximation of the degree of vacuolation is the total lesion score and is obtained by summing the average values of the 9 individual lesion scores. All sections are examined and scored 'blind' and in random order.

Lesion profiles are based upon grey-matter vacuolation. Some agent/host strain combinations also show a degree of white-matter vacuolation which is referred to as "oedema" for simple distinction. It is possible to distinguish with a high degree of efficiency between scrapie lesions and oedema due to age.

Reference standards for incubation periods and lesion profiles, using the methods outlined above, are now available for many combinations and are based upon results from a total of more than 25,000 mice.

SECTION 3

DEVELOPMENTAL MATURATION OF SUSCEPTIBILITY TO SCRAPIE IN MICE

DEVELOPMENTAL MATURATION OF SUSCEPTIBILITY TO SCRAPIE IN MICE

When newborn mice are injected by the i.c. route with standard supernates of ME7 scrapie, the incubation period is virtually the same as that after the same dose and route of injection in weanlings, because the reduction of incubation period with increasing age at injection is very small: $b = -0.05 \pm 0.01$ days/day over the range 0-130 days of age for the ME7 agent in C57 mice (Dickinson & Meikle, 1971). To the extent that shorter incubation periods are an indication of greater susceptibility, this is an unexpected result, since one would normally expect neonates to be more rather than less susceptible to an infection than older animals. This negative regression is manifest moreover, even though the neonates receive a much higher dose in proportion to body weight than weanlings. However, when newborn mice are injected with scrapie using the i.p. route, very different results are obtained from those of weanlings. Previous to Outram, Dickinson & Fraser (1973) other published attempts to investigate the effect of age upon the incubation period of peripherally injected scrapie agent showed that no difference could be detected using mice as young as 6 days old (Kimberlin & Millson, 1972). When, however, standard scrapie supernates are injected by the i.p. route into mice during the first three days of life, the incubation periods are generally very greatly lengthened, with a proportion of animals that do not get scrapie at all. (There is in addition a small proportion of mice with incubation periods that are significantly shorter than those amongst weanlings: an untidy-looking feature which may, however, be important for a proper understanding of the phenomenon).

Special procedures for injection in neonates

For the injection of newborn mice a number of special precautions were taken in order to minimise the possibility of losing inoculum by leakage. When animals were less than a week old at the time of injection, a fine (32G) hypodermic needle was used and inserted into the peritoneal cavity from a point of entry near the liver having run a short way subcutaneously from near the xiphisternum. The injection site was then sealed with Nobecutane acrylic solution.

Ex. 3.1 Tables 3.1 and 3.2 report the results of using three agents (ME7, 79A and 22A) in three strains of mice (C57, VL and VM) with ages ranging from birth to 28 days (weaning normally being on the 21st day). Three different concentrations of standard supernates of infected brain were used in the case of ME7: 10^{-1}, 10^{-2} and 10^{-3}, the others used 10^{-2} only.

Results

Because the means and variances of the incubation periods in the different agent/host strain combinations differ so much from each other the results have been expressed in Table 3.2 in terms of Standard Deviations (SD) on either side of the mean incubation periods, in weanlings, for each agent/host strain/sex combination. For more efficient estimates of these standard deviations than could be obtained by using only the results from the weanlings in these experiments, they were calculated after adding the values of incubation periods from other comparable non-contemporary replicates. These pooled

results are shown in Table 3.1. The incubation periods of the contemporary and non-contemporary weanling control groups were closely similar to each other.

Table 3.1 The incubation periods shown by weanling mice after an i.p. injection of various strains of agent

Agent	Mouse strain	Dilution of infected brain	Number of mice	Incubation period	
				females	males
22A	VM	10^{-2}	27	319±6	355±5
79A	VL	10^{-2}	38	205±1	215±2
ME7	C57	10^{-1}	15	225±4	233±3
		10^{-2}	36	249±2	250±4
		10^{-3}	11	278±6	275±3

However, it can be seen that in the case of mice that were injected in the first few days of life, numbers of individuals showed very wide deviations from the mean. There is a complication, however, due to an interaction with dose as indicated by the ME7 results. These suggest that cases following a neonatal injection tend to occur earlier than weanlings with the high titre inocula, but later with the low titre inocula. In accord with this trend, survivors only occurred in the newborn group of C57 mice when given the lowest titre of ME7 agent. The proportion of survivors using 10^{-2} homogenates was higher in the case of the other two agent/host strain combinations than for the ME7/C57 combination. Animals were regarded as 'survivors' if they either died or had to be killed without clinical signs of scrapie at more than 550 days old.

Table 3.2 The distribution of incubation periods of three scrapie agents injected i.p. in mice of different ages. The individual incubation periods are classified in terms of standard deviations from the mean incubation period in weanling mice

Agent	Mouse strain	Dilution of infected brain	Age (days)	n	Numbers of individuals with incubation periods in SD groups less than (-) or more than (+) the mean for weanlings					
					->3 SD	-3 to 2 SD	-2 to +2 SD	+2 to 3 SD	+>3 SD	survivors†
22A	VM	10^{-2}	21-28	27		2	24	1		
			4-6	16	2		9	1	1	3
			1	8						8
			0	10	1					9
79A	VL	10^{-2}	20-28	37		1	34	2		
			6-7	28		6	15	5	2	
			2-3	15			5	6	4	
			0-1	24	1	1		2	11	9
ME7	C57BL	10^{-1}	21-28	8		1	7			
			7-8	6	2		4			
			0	17	7	3	2		5	
		10^{-2}	21-28	20			19	1		
			4-8	31	3	4	10	5	9	
			2	7	1	3	2	1		
			0-1	31		4	18	1	8	
		10^{-3}	21-28	11			10	1		
			8	8			2	1	5	
			3	5					5	
			0	6	1			1	2	2

† animals injected with scrapie but dying or having to be killed for clinical conditions other than scrapie at more than 550 days old.

SD = Standard Deviation.

Depending on the particular agent/host combination this age represents individuals with incubation periods more than 10 to 50 SD later than the mean for weanlings. In addition, these survivors were found histologically to have no scrapie lesions.

Ex. 3.2 Modification of the neonatal age-effect in VL mice with 79A scrapie by the addition of adult bone-marrow and thymus cells to the inoculum.

As a test of whether or not the lymphoreticular system is involved in the neonatal age-effect, an attempt was made to modify it by giving mice in the age-range 0-20 days a scrapie inoculum i.p. to which had been added living bone-marrow and thymus cells. This type of mixture of B- and T-cells has frequently been shown to be efficient in the restoration of immunocompetence to mammals after various kinds of experimental manipulations like thymectomy, sub-lethal irradiation, tolerogenic regimes etc. (Claman & Chaperon,1969; Playfair, 1968; Miller & Mitchell, 1968; Mitchell & Miller, 1968; Nossal et. al., 1968). Such a mixture, which was not washed, probably also contained some physiologically active substances like thymosin (Trainin et. al., 1969) but these would be rather unlikely to have any effect in a single small dose.

Procedures The cell suspensions were prepared by washing out the contents of both femurs of 3 weanling and 4 five week-old VL female mice using 2 ml. of ice-cold Hank's balanced salt solution (HBSS). 1.75 ml. of this suspension was mixed immediately before injection with the same volume of a thymus cell suspension prepared by gentle teasing

in another 2 ml. of cold HBSS and taken from the same animals. Viability, using the Trypan blue exclusion test was 80%.

This cell suspension was then mixed with an equal volume of a 2×10^{-2} standard 79A brain supernate which had been prepared from a terminal RIII female brain using HBSS for suspension instead of saline.

62 female and 61 male VL mice ranged from 0-21 days old were injected i.p. with 0.02 ml. doses of this mixture, taking the usual precautions against loss of inoculum in those less than one week old. Contemporary controls were 5 ten-day old and 3 four-day old VL mice injected i.p. with 0.02 ml. of the 79A supernate diluted with HBSS only (i.e. no cells). (Also some mice were injected with the cell suspension only to check for the absence of infection in these inocula).

Results The incubation periods of the controls indicated a normal titre of 79A agent and therefore validated a comparison with large non-contemporary groups of VL mice which had been given 79A i.p. and covering the same age-range but without any cell-treatment. The percentage incidence of individual incubation periods in the standard deviation groups about the means of VL mice of different ages are given in Table 3.3.

Table 3.3 The incubation periods of 79A i.p. in VL mice of various pre-weaning ages, with[a] or without[b] the addition of lymphoreticular cell suspensions.

Age range (days)	n	% individuals in each SD group					
		>-4SD	-4SD to -2SD	-2 to +2SD (i.e. 95% in weanlings)	+2 to +4SD	>4SD	Survivors
18-20	12[a]	17	25	58	0	0	0
	42[b]	0	5	90	5	0	0
6-15	42	5	14	81	29	0	0
	45	0	16	58	27	0	0
3-5	20	10	0	30	60	0	0
	16	0	0	62	31	6	0
0-2	26	22	6	22	39	11	0
	33	6	3	21	21	24	26

[a] The first line in each age group gives the values after cell-treatments.

[b] The second line gives values without cell-treatments.

This comparison of the incubation period distributions obtained in age-experiments with and without treatment with adult lymphoreticular and haemopoietic cell suspensions shows that such treatment reduces the neonatal resistance to peripherally injected scrapie and increases the proportion of early cases. These effects are most apparent in the first two weeks of life, but continue less strongly up to the time of weaning.

It should now be possible to identify the particular factor or factors responsible for this effect. Clearly it could be due to a number of possible causes such as, a general activation of the neonatal peritoneal cell populations by the adult tissues, or the addition of particular cell populations, missing from the newborn mouse but required directly for scrapie pathogenesis, or a premature development of the necessary cells brought on by hormones in the inoculum (e.g. thymosin, Trainin et. al., 1969). A rather less interesting possibility is that a hastened maturation may have been caused by a mild infection due to some conventional virus in the inoculum, although no sign of such an infection was seen in the animals injected with bone-marrow and thymus cells only.

There is no way yet of choosing between these possibilities, although my inclination, in view of the involvement of the agent with lymphoreticular tissues, is to suspect that the treatment worked by contributing one or more of the necessary cell populations. Further experiments using sub-populations (and later perhaps, mixtures of sub-populations), cell-free extracts and tissues from other organs, are in preparation.

A small experiment already performed is of interest in this context. Adult peritoneal macrophages (stimulated by thioglycollate 8 days previously) were given i.p. to a newborn litter of VL mice two days before an i.p. injection of 79A scrapie. This treatment apparently prolonged the incubation period (247 ± 6 days) beyond what is usual for this age (227 ± 5 days) $P = 0.02$, with the production of two survivors which were negative both clinically and

histologically at 600 days. If this observation is substantiated on a larger scale and using more elaborate controls then it will be evidence for a scrapie inactivation capacity of macrophages.

SECTION 3 DISCUSSION: INFLUENCE OF THE STATE OF DEVELOPMENT ON THE INCUBATION PERIOD OF PERIPHERALLY INJECTED SCRAPIE.

The range of results in this SECTION could be important for the future investigation of the pathogenesis of scrapie in mice. There are several possible explanations for them such as the production of some form of neonatal tolerance, or massive excretion of the agent, but the most likely is probably that neonatal mice lack one or more of the cell populations that are required for the initiation of scrapie pathogenesis by the peripheral route. The possibility of tolerogenesis is examined in SECTION 4 but no evidence of it has been found.

Hypotheses about the cause of this neonatal age-effect on the basis of cellular immaturity need to account for the disposal of agent activity before the relevant cells develop and for the observation that early cases do in fact occur, in line with what one would expect in conventional virus infections, and this can apparently be encouraged by simultaneous injections of adult bone-marrow and thymus cell suspensions, or by using higher titres of agent. In other words, they must account for the paradox that the incubation period data in neonatal mice would give lower estimates of titre than in weanlings, but with shorter incubation periods than weanlings at the higher doses. Complete titrations at different ages are at present in progress in these laboratories and will therefore provide more

adequate data to illustrate this point.

Perhaps the simplest theoretical condition to meet the above requirements is that there are at least two sets of relevant cell types (or systems within one cell) that are developing at different rates in newborn mice, and that individual differences in their relative maturation at this unstabilised stage are responsible for individual variations in incubation period. Thus, if there were two, antithetical conditions in the host - one favouring the replication of the agent and the other causing a loss of activity - then individual differences in the rate of development of one or other would interact with the concentration of agent administered to give a wide array of incubation periods ranging from the very short to the very long as compared with those of mature animals in which the proper balance of functions had been achieved.

At a higher level of complication the requirements referred to are met by a model employing three sets of relevant cell types: the above mentioned two and a third with the role of transporting the agent from the first to the second - a process which is normally accomplished before there has been much inactivation. Immaturity of this type of cell in neonates would also facilitate inactivation and frustrate replication of peripherally injected agents, while premature development could result in shorter incubation periods than in weanlings.

The necessity to postulate some system for the inactivation or excretion of the agent comes from the known high resistance to physical or chemical inactivation of scrapie (Stamp, 1967) which would otherwise lead one to expect that agent could persist - for weeks if

necessary - in the body of the young recipient, until the necessary receptors matured and replication could commence. While something of this sort would clearly account for prolonged incubation periods such as occur, something else must be involved in the case of survivors. There is no realistic possibility that these results could be due to the loss of the inoculum by leakage, because the values for the long incubation periods would require the loss of nearly all of it and specific precautions were taken. If it is correct, therefore, to conclude that one or more relevant sets of receptor cells for replication are absent in the newborn mouse, three possibilities must be considered for the fate of the agent. It could be effectively disposed of by excretion (e.g. through the gut wall which is permeable to several macromolecules at this stage), or by sequestration in some alternative tissues where it cannot replicate, do damage or gain access to target cells, or thirdly, the agent could be inactivated enzymically, for instance by macrophages which presumably have somewhere in their armoury a means of dealing with any organic molecule. Excretion through the gut wall is perhaps the least likely and is argued against by results described in SECTION 4 where it is shown that large doses of steroids can potentiate the neonatal age-effect. Such doses of exogenous steroids are known to cause a premature closure of the gut to macromolecules which would presumably apply also to scrapie (Daniels & Hardy, 1972).

This potentiating effect of steroids also supports the other two possibilities: sequestration and destruction by macrophages. With this type of model, the longer incubation periods and the

survivors amongst neonates would be accounted for by postulating that the disposal system normally developed before the one favouring replication, so that with sufficiently low titres of agent most or all activity could have been removed before the scrapie-supporting tissues had time to mature. Support for the notion of a peripheral system inimical to scrapie replication also comes from the operationally lower estimates of titre obtained in mice injected after weaning by the i.p. route as compared with the i.c. one.

Of the many systems that are still developing in the neonatal mouse the lymphoreticular/haemopoietic system is a very likely one to be involved. Other studies on the age-dependent susceptibility of mammals to virus infections have implicated various cells of this system, particularly the macrophages (Johnson, 1964; Goodman & Koprowski, 1962; Gallily et. al., 1967; Kantoch & Dobrowolska, 1969; Hirsch, Zisman & Allison, 1970). The contrast must, however, be emphasised between these systems, where the immaturity of components of the lymphoreticular system renders the individual generally more susceptible, and the case with scrapie where the reverse applies, and in which, if these tissues are involved they would assume the role of a Trojan horse in which the agent could replicate and perhaps be transported unhindered and undetected by the host.

The normal development of the lymphoreticular system is also related to the complex process of the acquisition of immunological competence (Argyris, 1968; Tyan, 1968; Tyan & Herzenberg, 1968; Claman & Talmage, 1963; Playfair, 1968a). In most age-dependent

susceptibility situations it is possible to explain the findings in terms of the involvement of the normal immune response, but this seems most unlikely in the case of scrapie to which no specific immune responsiveness has ever been certainly demonstrated. It is possible that scrapie agents may be capable of using some kinds of weak host immune responses without provoking reactions specifically against themselves. SECTION 6 describes a system in which antigens probably of donor-origin in the homogenate may be able to influence the course of pathogenesis in such a manner as to suggest that the agent is making use of specific host responses to these donor antigens.

An important phenomenon which this neonatal age-effect may eventually help to explain is that while there is clear evidence for maternal transmission of agent in sheep (Gordon, 1960; Dickinson, 1967; Dickinson, Young, Stamp & Renwick, 1965) there is considerable evidence against its occurrence in mice (Clarke & Haig, 1971; Dickinson, unpublished). It may be significant that while sheep become immunologically responsive to several antigens many weeks before birth (Schinckel & Ferguson, 1953; Prendergast, et. al., 1969) mice are immunologically unresponsive at birth. Could the difference in maternal transmission of scrapie in the two species be due to foetal sheep having the relevant scrapie receptors somewhere in their lymphoreticular systems, while mice do not develop them until a few days after birth?

There is also the possibility that an understanding of the apparent ability of hosts to dispose of scrapie activity under certain

circumstances could lead to methods of control.

SECTION 4 describes a drug-treatment which seems to mimic the age-effect in some respects, and further discussion of the possible role of the lymphoreticular system will be left until the discussion of those results.

SECTION 4

PHARMACOLOGICAL MODIFICATION OF SCRAPIE INCUBATION PERIODS

PHARMACOLOGICAL MODIFICATION OF SCRAPIE INCUBATION PERIODS

In accordance with the policy of trying to detect critical phases in the pathogenesis of scrapie agents by looking for ways to modify their incubation periods, particularly after peripheral injections of agent, a range of pharmacological treatments has been tested by means of pilot experiments. Of the several positive findings, one has been developed as a series of second generation, and to a certain extent third generation experiments. Drug treatments that gave negative results in pilots were not investigated further, although in the light of the positive results to be described, some of them will need to be re-examined using different doses and regimes.

Two prominent considerations attach to designing experiments for the investigation of scrapie pathogenesis by pharmacological means. The first concerns the choice of drugs, and the second relates to the dose, regime and timing of treatment with respect to the scrapie injection.

As to the choice of drug, this must be determined by what seems at the time to be the nature of the agent and its manner of interaction with the host. Scrapie agents apparently replicate peripherally in the tissues of the lymphoreticular system where the time of the onset and possibly the rate of replication is under the close control of the sinc gene. (Dickinson & Fraser, 1969). One choice of drugs therefore is that of substances known to upset normal lymphoreticular functions for which various immunosuppressants are obvious candidates. Amongst these are drugs which interfere with

normal nucleic acid metabolism and although the question of whether or not scrapie agents contain nucleic acids has not been settled, they clearly could do so, and in any case may depend fairly directly upon the normal function of host nucleic acids for their own replication and/or pathogenic action.

The question of the correct dose, regime and timing with respect to the scrapie injection arises because of the unusually long incubation periods as compared with those of conventional pathogens. Most of the published data on the doses of relevant drugs in mice are based upon short-term treatments after which the animals are usually killed, so that long-term effects are often not known. For example, Stenram & Nordlinder (1968) have shown that if, instead of being sacrificed quickly, rats are kept after treatment with cyclophosphamide (65-110 mg/kg body weight) they usually die after about three months with a wasting disease. Long-term treatments with drugs have other disadvantages. It is difficult to judge the level of dose which might be enough to modify scrapie pathogenesis but which will neither harm the host nor allow it to develop effective detoxicating enzymes or antibodies. The most hopeful type of treatment which might change the incubation period would be one in which as high a dose as possible was given for a period of two or three weeks at a time critical for agent replication. Two such periods are probably relevant: one shortly after a peripheral injection when a relatively rapid increase in titre occurs in lymphoreticular tissues, and a second about half way through the incubation period when there is a sudden change in brain metabolism accompanied by the appearance of

lesions and a clearly detectable rise in titre. (Numerous references reviewed by Kimberlin, 1973). An interference with agent replication by a short burst of drastic treatment at the beginning of the exponential phase of increase would be more likely to be projected as a change in incubation period than would an attempt to interfere continuously with replication using chronic treatment at lower doses.

The practical question therefore, of what doses to use in pilot experiments was answered by using published dosages as a guide and then increasing or decreasing them appropriately to suit a course of two or three weeks intensive treatment, and finally monitoring the mouse response by taking body weights and observing behaviour generally. This procedure sometimes resulted in short-term or long-term death from toxic effects, and sometimes in an underestimation of the dose required to affect scrapie despite this general approach. As will become apparent in the account of the experiments involving prednisone acetate the question of dose can be very critical indeed and it may be that some of the negative results listed in Table 4.1 reflect a failure to find the correct regime rather than an inefficacy of the drug.

RESULTS OF EXPERIMENTS SHOWING NO EFFECT OF DRUG TREATMENTS ON SCRAPIE INCUBATION

Ex. 4.1 Drug treatments that showed negative results are listed in Table 4.1. These are dismissed briefly since it would be tedious to describe them in full only to be left with uninterpretable negative results.

Drug	Outline of regime and dose	Route of injection of 79A	Mouse strain
Streptomycin	2ug i.p. 2 x week for 5 weeks, starting 7 weeks after 79A	i.c.	A2G
	1mg/mouse/day i.p. 25 x in 5 weeks after 79A	i.p.	BALB, RIII
Magnesium pemoline	25mg/kg in 100% DMSO i.p. daily for 3 weeks after 79A	i.p.	C3H, A2G
	25mg/kg in 100% DMSO i.p. 3 x week from 60-90 days after 79A	i.c.	A2G
Rifampycin	Estimated 50mg/kg/daily oral in drinking water throughout incubation	i.p.	C3H, A2G
Iododeoxyuridine	250ug/mouse/day 2 x week for 5 weeks after 79A	i.p.	Porton mice
Salicylhydroxamic acid	10mg/mouse/week in DMSO for 5 weeks after 79A	i.p.	SM, LM (all died)
6-mercaptopurine	200ug/mouse/day i.p. 10 x in 2 weeks	i.p.	SM
	200ug/mouse/day 25 x in 5 weeks, starting 7 weeks after 79A	i.c.	A2G
Caffeine	1mg/kg i.p. 20 x in 4 weeks after 79A	i.p.	Porton mice
Actinomycin D	1ug/mouse/day i.p. 10 x in 3 weeks starting 2 days before 79A	i.p.	BALB, RIII, SM, C3H
Cyclophosphamide	2.5mg/kg daily for 2 weeks	i.p.	C3H
	100mg/kg i.p. 3 days before 79A; 6 daily prednisone 50mg/kg starting 7 days after 79A	i.p.	RIII

PREDNISONE ACETATE (PA)

Very marked effects upon scrapie incubation periods have been obtained using this drug and most of this SECTION will be devoted to describing experiments which explore this effect. A single subcutaneous (s.c.) injection of cortisone acetate (400-500 mg/kg) in 8-12 week-old Swiss white mice depresses the serum haemolysin response to sheep erythrocytes when administered near the time of antigen injection, but especially 3-4 days previously. It causes a reduction in the levels of antibodies (both 19S and 7S); in the numbers of circulating lymphocytes and monocytes; in the weights of the thymus, spleen and lymph nodes. Polymorphonuclear leucocytes (PMN) fall to 25% of normal on the 1st day, recover to control level by the 4th day, overshoot to twice the control count by the 10th day, then return to normal by the 40th day (Elliott & Sinclair, 1968).

In view of the association of peripheral scrapie replication with the lymphoreticular organs it was decided to base a pilot experiment upon this type of protocol. In addition, although it seemed possible that a single injection as described would be sufficient, it was decided to try to maintain the depressed state produced by the large dose by adding a follow-up course of steroid injections at a lower individual dose. A potentially better steroid than cortisone for this sort of treatment is the artificial glucocorticoid, prednisone acetate (1,4-pregnadien-17,21-diol-3,11,20-trione 21 acetate). This has about three times the glucocorticoid effects of the natural steroid but with greatly reduced mineralocorticoid effects and thus is more suitable for large and prolonged doses.

Ex. 4.2 Prednisone acetate pilot experiment

15 adult VL mice were treated with PA or saline before and after an i.p. injection of standard 79A frozen brain supernate. Three days before the scrapie injection the 9 experimental animals received 250 mg/kg PA in 0.1 ml. saline s.c. Then on days 18, 21, 25, 28 and 32 after the scrapie injection they received 12.5 mg/kg doses of PA s.c. The 6 control mice were given saline injections s.c. There was no further treatment and the disease was left to run its normal course. All the mice developed scrapie and their incubation periods were as follows:

Table 4.2

Group	Incubation period	Range (days)
Controls (saline-treated)	209 ± 2	206-239
Experimentals (PA-treated)	273 ± 39	204-527

Three of the experimental animals showed incubation periods within the range of the controls, but five of them had considerably prolonged incubation periods. This was therefore a very promising result although the high variance ratio prevented an assessment of significance using simple tests (Snedecor, 1946).

The most immediate question to arise was whether the steroid acts by interfering in some way with agent replication or by changing the threshold of host responses so that it can survive much higher levels of agent concentration in its tissues. Accordingly the

spleen was removed from one of the females in this experiment, killed with no clinical signs of scrapie 338 days after the scrapie injection. A 10% saline spleen homogenate was prepared and injected without centrifugation by the i.c. route into C57 mice: none of these had developed scrapie 680 days later and were also found to be negative histologically. This result suggests that the steroid interferes with the initial stages of peripheral pathogenesis so that there is a failure on the part of the agent to reach some or all peripheral replication sites. This resembles closely the demonstration by Nettesheim and Hammons (1970) that several immunosuppressants, including cortisone acetate interfere with the normal accumulation of peripherally injected antigens in the spleen.

It should be appreciated that a period of over 1000 days had lapsed between the start of the PA pilot and the final demonstration of the absence of scrapie in the C57 assay animals. In the meantime a considerable programme of follow-up experiments was mounted using the tactical approach outlined in SECTION 1. These will now be described in an order chosen for clarity rather than historical accuracy. As a result it will sometimes appear that certain inappropriate experimental procedures were still being used long after they should have been dropped, because early experiments are being reported after later ones. One particularly striking illustration is perhaps best described at this juncture. As a result of the high variance in the incubation periods of the pilot experiment it was decided to suspend the PA in arachis oil, rather than in saline, in order to ensure uniformity of dose in case this was the cause of the variation in response. Arachis oil was chosen since it was in use

in commercial preparations of other steroids. Many of the follow-up experiments employed this 'neutral' vehicle and it was not until a year and a half later that it became apparent that the arachis oil itself had a marked prolonging effect on incubation periods of peripherally injected scrapie. It was immediately replaced by 2% Tween 20 in saline as the suspensory medium, and of course, became a substance of interest in its own right. However, this was not discovered before it had rendered several experiments partially uninterpretable at least in the terms under which they had been designed.

Ex. 4.3 An attempt to repeat and extend the PA pilot result including a test of its effect on i.c. injected agent.

This repeat used larger and more intensive courses of injections of PA, slightly older animals and higher doses of agent than the pilot. Some animals received the scrapie injection by the i.c. route.

13 and 6 female VL mice received, respectively 0.1 ml. i.p. or 0.02 ml. i.c. of standard 79A supernate. The mice were treated with PA in saline or saline only (controls) in the following manner. Four days before the scrapie injection 7 of the females (to be given i.p. scrapie) and 3 of the females (to be given i.c. scrapie) were injected s.c. over the shoulder with the equivalent of 500 mg/kg of PA in 0.1 ml. saline. Beginning seven days after the scrapie injections these animals were given 11 daily equal doses of 100 mg/kg each of PA s.c.

Incubation periods were as follows:

Table 4.3

Group	Incubation period	P
i.p. injected controls	184 ± 3	<0.02
PA-treated; 79A i.p.	207 ± 8	or
	or	
	* 212 ± 6	<0.01
i.c. controls	148 ± 6	>0.1
PA-treated; 79A i.c.	148 ± 6	

* omitting a single early value of 179 days

These results show no evidence that intensive courses of PA at the time of injection can influence the pathogenesis of i.c. injected agent. The incubation periods of the i.p. injected group confirm the pilot results and on this occasion the variance ratio of the two groups is low enough to permit the calculation of the significance of the effect using a simple t test. The effect of the PA on incubation period was, however, less marked than in the pilot despite the much higher doses. The two protocols differ in the following respects:

- the mice in the pilot experiment were 32 days old, and those in the repeat 35-60 days old at the time of scrapie injection;

- the timing and dose of steroid was different, with a total of 312.5 mg/kg in 5 weeks in the pilot, and 1600 mg/kg in 3 weeks in

this experiment;

- the incubation periods of the controls indicate that the titre of the 79A in the frozen supernate material used in the pilot was about 1.5 log LD_{50} units lower than that of the brain pool material used in this experiment.

The previous SECTION showed how age of host and titre of agent can interact in a complex way to influence the incubation period and it is possible that something of the kind is happening here too.

The lack of effect on i.c. injected agent could be due to either (a) failure of the steroid to reach the necessary concentrations in the brain in order to react effectively with either the agent or the relevant host mechanisms, or (b) the absence from scrapie pathogenesis in the brain immediately after an i.c. injection of one or more steroid-sensitive steps [illegible] obligatory in peripheral pathogenesis.

Ex. 4.4 Three experiments to investigate the effect of using very young (unweaned) VL mice in PA experiments

It was appreciated quite early in the series of PA experiments that the drug appeared to produce in adults an effect on incubation periods similar to that of the neonatal state. Three early experiments investigated the effect of using the original PA regime on unweaned mice. All three used frozen homogenates of agent as was the practice with small groups of animals (see SECTION 2).

Litters of VL mice aged 3, 6 and 13 days old were injected s.c. with 250 mg/kg doses of PA in saline or with saline only. Four days later all mice received i.p. injections of 0.02 ml. standard

79A frozen brain homogenate. Then, beginning 17 days later, the experimental groups received 6 follow-up doses, each of 12.5 mg/kg PA in saline, on days 17, 20, 24, 27 and 31.

Incubation periods were as follows:

Table 4.4

Age at i.p. scrapie injection (days)	Group	Incubation period of controls	Individual incubation periods of PA-treated mice
17	Saline-treated controls	204 ± 6	
	PA-treated		219,231,480,580
10	Controls	224 ± 7	
	PA-treated		482,486,600*,600*
7	Controls	213 ± 5	
	PA-treated		166,166,183,315,700*

* clinically negative animals found to be histologically negative

These results show that unweaned mice are more sensitive to the effects of this PA regime than weaned mice are, even to the extent of producing survivors among mice injected at 10 days of age. (cf SECTION 3). The very great spread of incubation in the 7 day olds was, however, quite unexpected, and since at the time we were not yet reconciled to its occurrence in the neonatal age-effect the result seemed to be anomalous. However, as will be illustrated by other experiments in this SECTION, such very early cases are a feature of the PA-effect in very young mice and may be caused by a steroid-induced physiological state similar to the one in

neonates which also results in very short incubation periods as compared with weanlings.

Ex. 4.5 Two experiments to investigate the effect of varying the size of the initial dose of PA on the incubation period of 79A in weanling VL mice

Some of the early follow-up experiments were attempts to see if large PA injections given a few days before the scrapie injection were in fact all that was necessary (as in the immunological 'model' of Nettesheim and Hammons, 1970) and if so, what dose would be necessary. These experiments also used arachis oil as a vehicle.

In one experiment, VL mice were given 1-3 i.p. injections each of 500 mg/kg PA suspended in arachis oil during the three days preceding a standard 79A injection i.p. In another experiment, they received either 500, 250 or 50 mg/kg PA in arachis oil i.p. three days before the standard 79A injection i.p. Thus the PA dose in these two experiments ranged from 50 to 1500 mg/kg immediately before the scrapie injection. In the second experiment some controls were injected with oil and the others were completely untreated. Neither experiment employed maintenance doses of PA.

The incubation periods were as shown in Tables 4.4 and 4.5:

Table 4.4

Group	Incubation period
Three pre-treatments with oil-only i.p.	197 ± 1
500mg/kg PA in oil i.p. on day -1	197 ± 2
Total of 1000mg/kg PA in oil on days -1 & -2	*181 ± 16
Total of 1500mg/kg PA in oil on days -1, -2 & -3	200 ± 0

* including one very early case at 150 days with good lesions

Table 4.5

	Uninjected controls	Arachis oil controls	PA in arachis oil		
			50mg/kg	250mg/kg	500mg/kg
Females	185 ± 2	195 ± 4	195 ± 3	199 ± 7	197 ± 4
Males	194 ± 8	197 ± 7	214 ± 4	206 ± 7	216 ± 4

The probability that the difference between the incubation periods of the female uninjected controls and the females pre-treated with arachis oil is due to chance is greater than 5%, and the addition of PA did not increase the significance of the difference, but the trend is consistent. Similar conclusions apply to the male results.

These values show that single injections of PA in oil i.p., even when very large doses are used do not greatly affect the incubation period of 79A in weaned VL mice. Some of the problems in

this kind of work are well illustrated in this experiment. The emergence of a large sex difference in the second experiment was not anticipated at the time of the design and resulted in a great reduction of the intended group size. Also, no significance was attached at this stage to the arachis oil control results so that this vehicle continued to be employed in subsequent experiments.

Ex. 4.6 Two experiments using a testosterone treatment of VM mice with 22A agent to see if it would increase the sex-effect and/or produce results similar to PA

The agent/host strain combination of 22A agent in VM mice using the i.p. route of injection was discovered to show a marked sex-effect with the males having a longer incubation period than females (Dickinson & Meikle, 1969 and APPENDIX 3). Experiments using sex-steroids were therefore undertaken to see if they would throw any light on the matter and also to see if steroids other than glucocorticosteroids would also affect scrapie incubation periods, especially since the sex steroids also have effects on the size and immune functions of the lymphatic system (e.g. Frey-Wettstein & Craddock, 1970). The results of these experiments will be described very briefly only since no effect of sex-steroid treatment was detected but they provided the first clear indication that arachis oil has an independent effect of its own.

In the first experiment, VM mice were treated with courses of testosterone phenylproprionate in arachis oil, or follicle-stimulating hormone (FSH) in saline, or saline only over a period of three weeks straddling a mid-course i.p. injection of 22A agent. The incubation

periods are shown in Table 4.6.

Table 4.6

	Incubation period				
	Saline controls	FSH treated	P	Testosterone treated	P
Females	295 ± 3	300 ± 4	>0.1	323 ± 8	<0.01
Males	313 ± 8	310 ± 9	>0.1	353 ± 6	<0.01

The FSH-injections had no effect on the incubation periods in either sex. The testosterone result, however, seemed at the time to be highly significant. Its effect had been detected several weeks before the end of the experiment by a delay in the time of loss of body weight in these groups (APPENDIX 1) suggesting that the onset of some stage of pathogenesis had been postponed a few weeks. However, it was not possible to discount arachis oil as the active ingredient, although this seemed very unlikely at the time. (Previous experiments in these laboratories using i.p. injection of ethylpalmitate had not produced significant changes in incubation periods which might result from lipid blockade of macrophages etc.: Fraser, pers. comm.).

In the second testosterone experiment using female VM mice infected with 22A agent and similarly treated with oil suspended testosterone also included both arachis oil-treated and untreated controls. The incubation periods were as follows:

Table 4.7

Group	Incubation period	P
Untreated females	317 ± 2	0.05-0.02
Arachis oil-treated females	338 ± 8	
Testosterone-treated females	348 ± 11	>0.1

This result shows clearly the effect of the arachis oil alone. Although the mean incubation period of the steroid-treated group is longer than that of the oil-treated controls the high variance ratio makes a significance test unreliable.

Ex. 4.7 An experiment to test the effect of a single large PA injection on the day of birth on the incubation period of 79A injected i.p. a week later

The 7th day of life lies outside the age range for the production of survivors from i.p. injections of standard supernates of 79A scrapie in untreated VL mice (SECTION 3). The neonatal age-effect could be due to the post-natal depression of lymphoreticular tissues associated with changes in glucocorticoid levels in early post natal life (Burton & Jeyes, 1968; Philpott, Zarrow & Deneberg, 1969). If so, an injection of a depot of PA may be able to maintain this state for longer than just one or two days and produce survivors in older animals.

A newborn litter of 5 female and 5 male VL mice was injected

i.p. with either 500 mg/kg dose of PA in arachis oil or were uninjected. This dose profoundly retarded normal body growth and caused hair curling as has frequently been described in rats (e.g. Field, 1957; Shapiro, 1965). At 7 days after birth all the mice were injected i.p. with standard 79A frozen brain homogenate. Incubation periods were as follows:

Table 4.8

Group	Individual incubation periods	
	Females	Males
Untreated controls	223,230,230	244,244
PA/oil-treated	230	-
	one killed 556*	two killed 534*, 573*

* clinically and histologically negative

Independent titre control of this frozen homogenate indicated a 2 log drop as compared with usual preparations. Although the treatment would appear to have been effective in producing survivors it is not possible to separate the component effects of age, agent titre, arachis oil and PA.

Ex. 4.8 An experiment to investigate the effect of a single large injection of saline-suspended PA, injected s.c., on the incubation period of 79A injected i.p. in VL mice over a wide age range

135 female and male VL mice ranging in age from birth to 25 days were injected i.p. with 0.02 ml. standard 79A supernate.

The usual precautions were taken against leakage in animals less than one week old (SECTION 3). Four days before this injection the mice then aged from 2 days upwards were given 50 mg/kg PA in saline, or saline only, by the subcutaneous route. No maintenance doses were given. Animals were weaned at 21 days.

Incubation periods are shown in Table 4.9.

Table 4.9

Age at 79A injection i.p. (days)	Controls		PA-treated		Probability of difference due to PA-treatment	
	females	males	females	males	females	males
0-1	263±37	248±4$^{\phi}$	-	-	-	-
6-11	219± 2	225±3	212±3	217±6	<.05	>.1
14-20	201± 2	211±6	212±2*	219±1$^{\phi}$	<.01	>.1
22-23	202± 1	213±5	206±2	207±6	<.05	>.1
24-25	208± 3	215±4	203±2	209±3	>.1	>.1

ϕ * in addition there were 4 and 2 survivors respectively.

The controls in this experiment showed the typical age-effect with 79A in VL mice: the neonates having much longer average incubation periods, with a wide range of individual values and the occurrence of some survivors. The 6-11 age range had incubation periods intermediate between those of neonates and weanlings. The PA-treated results fall into three groups:

- the 6-11 day group in which the change of incubation period was in the direction of shortening (just significant in the females);

- the 14-20 day group in which incubation periods were prolonged (highly significantly in females) and with survivors in both males and females; and

- the 22-25 day (post-weaning) group in which there was no effect.

This experiment employed a single relatively low dose of PA in saline injected by the s.c. route. The shortening or lengthening effects of this injection according to age are probably the consequence of a complex interaction between age, steroid and agent dose and the details cannot be resolved at this stage. Indeed nothing short of full titrations in mice of both sexes, at various ages between 3 and 30 days, with and without PA-treatment at various dose levels would properly sort out any such interactions. (This would require 2500 animals and more than $2\frac{1}{2}$ years).

Ex. 4.9 An experiment to investigate the effect of various lengths of maintenance treatment with PA after pre-treatment with a single high dose of PA before a 79A scrapie injection

Experiments so far described illustrate some variations in the amounts and timing of follow-up maintenance doses, but no systematic attempt to vary this has been described. There are numerous possible variations in dose, time of onset, frequency of administration and period of exposure. In this experiment weanling female and male VL mice were used. They were given a single 250 mg/kg dose of PA, suspended in 2% Tween 20/saline, by the s.c. route 4 days before a standard 79A injection i.p. and then maintenance

courses, beginning 7 days after the scrapie injection, of 1, 2, 4 or 6 weeks duration. The maintenance doses were given 3 times a week and each injection was 25 mg/kg PA in 2% Tween 20. Two kinds of controls were included: completely untreated and animals given a full course of 2% Tween 20 in saline injections s.c.

Incubation periods were as follows:

Table 4.10

	Uninjected controls	Tween 20 controls	Duration of PA maintenance course			
			1 week	2 weeks	4 weeks	6 weeks
Females	192±4	192±5	201±7	206±1	210±3	211±2
P		>0.1	>0.1	<0.05	<0.01	<0.01
Males	198±5	201±4	229±0	213±6	*220±3	220±7
P		>0.1	<0.001	>0.05	<0.01	<0.05

* or 209±13 with P of difference = >0.1 if a single early case at 166 days with good lesions is included.

These results indicate that Tween 20 has no effect on its own. Although significant effects of the maintenance doses are shown there is no indication that very prolonged treatments have a clear advantage over short ones: indeed the largest effect was obtained with males after only one week of maintenance doses. In the case of the males also, there is the possibility that the prolonged courses began to encourage the production of early cases. This could be explicable in terms of a rebound phenomenon resulting from an underproduction of endogenous cortisone due to the lag in recovery of ACTH production after the artificial steroid treatment.

Evidence that this can happen is shown especially by the numbers of polymorphonucleocytes in the blood after a single dose of cortisone acetate (Nettesheim & Hammons, 1970; APPENDIX 2). Another possible cause of the small effects of the follow-up doses in this experiment, is the relatively high titre of the scrapie inoculum used.

Again, although there can be no doubt that follow-up doses are necessary for the prolongation of scrapie incubation periods in adult mice the exact dose and regime which would give the best results is not known and could be difficult to discover.

Ex. 4.10 Two experiments to test the generality of the PA-effect by seeing if it will influence the incubation periods of the ME7 and 22C agents after i.p. injection in VL mice

In the interests of minimising the variables as much as possible all the PA experiments until recently were done using the 79A agent. An important test of the potential significance of any scrapie finding, however, is its generality. The agents ME7 and 22C are quite different from each other and from 79A although all three have relatively short incubation periods in s7s7 mice. They differ from each other in origin, type of interaction with the p7 allele of the *sinc* gene, the distribution of grey-matter lesions and especially in white matter vacuolation which is intense with 79A, slight with ME7 and absent with 22C in VL mice.

Male VL mice were injected i.p. after weaning with 0.02 ml. doses of standard ME7 or 22C brain supernates. Four days previously they received s.c. doses of either 500 mg/kg PA in 2% Tween 20 or

Tween 20 vehicle only. Then, beginning two days after the scrapie injection they were given daily s.c. injections for 2 weeks of 50 mg/kg PA in Tween 20 (or Tween 20 only). During the third week an attempt was made to facilitate withdrawal by giving only two spaced injections of 50 mg/kg s.c.

Incubation periods in the two experiments were as follows:

Table 4.11

Group	Early individual incubation periods (>3 SD (from mean)	Incubation period	P	Late individual observation times and survivors (>5 SD (from mean)
ME7 controls	187	248 ± 5		-
ME7 PA-treated		[a]305 ± 10	<0.001	
22C controls	-	263 ± 4		-
22C PA-treated	-	301 ± 8	<0.001	[d] 394 *>450 *>450

* still not showing clinical signs after 450 days.

[a] includes 3 minimum estimated values for animals which were killed accidentally when still clinically negative at 328 days: the real values could well have been higher.

Amongst the ME7 group of agents, 79A shows two peculiar features: it produces the shortest incubation periods and causes an intense white-matter oedema, so it was important to discover if the

PA-effect was confined to animals injected with this agent. The above results show that it is not, and by demonstrating the wider generality of the treatment allow the possibility that it acts at a fairly primary level of pathogenesis. That the steroid is not merely affecting a feature peculiar to the 79A agent is supported too by the fact that the lesion profiles and white-matter oedema levels in animals that have had their incubation periods prolonged by the drug are similar to those of controls.

Ex. 4.11 Experiments to determine if the modification of scrapie incubation periods by PA when injected in very young mice is due to a permanent change in the host

If one assumes that the effect of PA on scrapie incubation periods is due to some modification of the host's physiology, then it is possible that a steroid treatment which in the adult results in a transitory change would in a neonate cause a permanent one, and that this is the basis of the greater sensitivity to PA treatment in younger animals. Two obvious examples of this kind of phenomenon are the permanent androgenisation of female rats as a result of neonatal injections of testosterone(Barraclough & Gorski (1961) and the well-known instances of neonatal tolerance to antigens and viruses (e.g. lymphocytic choriomeningitis virus, Wiegand & Hotchin (1961)) with or without the aid of immunosuppressants. The following three experiments all employed an early immunosuppressive treatment (with or without an early exposre to the agent also) followed by a later injection of standard supernates after weaning.

1. If the PA-effect in weanling mice is some sort of restoration in adult life of the relevant neonatal state for scrapie 'resistance', then it might be possible to retain this state by a series of PA-treatments between birth and weaning. Three VL litters were injected on days 0, 8, 15 and 29 with either 500 mg/kg PA in Tween 20 vehicle, or with vehicle only using the s.c. route. Then on the 32nd day all mice were injected i.p. with a standard 79A supernate.

Incubation periods were as follows:

Table 4.12

Group	Incubation period			
	females	range (days)	males	range (days)
Controls	171 ± 2	169-176	207 ± 1	204-211
PA-treated	177 ± 7	162-184	205 ± 4	176-240
P	>0.1		-	

The probabilities that these differences are due to chance is greater than 5%. The treatment seems mainly to have increased the variability of the incubation periods but without producing any survivors. The variance ratio indicates that the probability of the differences in variance being due to chance are less than 0.001.

2. Another test of the effect on incubation period of steroid treatments starting in the first week of life is interesting although confounded with arachis oil effects. Four VL litters, randomised by redistribution at birth, were used. Three of them were then treated

with PA or vehicle in the following manner: eight alternating s.c. and i.p. injections of either 50 mg/kg PA in arachis oil or oil only were given to the randomised litters on days 3-9 and 11; one litter received oil only while the other two received PA. The fourth litter was untreated at this time. Ten days after birth, the oil-treated, and one of the PA-treated litters received 0.02 ml. of standard 79A (frozen supernate). The other PA-treated litter was not injected. Then 49 days after birth this PA-treated litter and the 4th, untreated litter, were given 0.02 ml. of standard 79A supernate i.p. The incubation periods were as follows:

Table 4.13

Group	Steroid/oil treatments days 3-9,11	Treatment on day: 10	49	Incubation period	Range of cases	Late negatives: died, or killed without scrapie (observation time days)
10 day control	oil	79A	-	*241 ± 10	225-259	268,306,335,338 490,650
49 day control	-	-	79A	214 ± 2	202-222	-
PA/oil/early 79A	PA/oil	79A	-	365	365	372,490,534,555 591,650,650
PA/oil/late 79A	PA/oil	-	79A	211 ± 4	208-229	-

* only 3 cases.

It can be seen that the early PA/oil treatment had no effect on the later i.p. injection of 79A on the 49th day of life. Quite unexpected at the time of these results was the very profound effect of the oil-only on the incubation period of 79A given on the 10th day

of life. However, the PA/oil mixture seems to have been even more effective than oil alone in producing late cases and survivors.

3. A third experiment to investigate the effect of early steroid on subsequent 79A injection was as follows. Three VL litters were randomised at birth and immediately given a s.c. injection of 500 mg/kg PA in Tween 20/saline. The next day they had one of the following treatments:

- 0.02 ml. saline i.p. (Control 1)
- 0.02 ml. normal brain supernate i.p. (Control 2)
- 0.02 ml. of standard 79A supernate i.p.

All these animals were then given a second i.p. injection of standard 79A supernate when 40 days old. Incubation periods were as follows:

Table 4.14

Group	Incubation period (calculated from 2nd injection)
Pretreated with PA + saline i.p.	198 ± 2
Pretreated with PA + normal brain	194 ± 1
Pretreated with PA + 79A brain	195 ± 2

Neonatal treatment with PA together with an early exposure to 79A brain or normal brain antigens had no effect on the incubation period of 79A injected i.p. 40 days later ($P > 0.1$).

In none of these three related sets of experiments did the early treatment have any detectable effect on the incubation period

of 79A given later in adult life. This was true even when the early treatment was shown to be capable of modifying the pathogenesis of scrapie given on the 10th day of life. The prolongation of incubation periods by the arachis oil treatment is also evident.

Ex. 4.12 An attempt to increase the effect of PA on the incubation period of 79A agent in adult VL mice by adding an injection of cyclophosphamide

The use of several immunosuppressants in sequence in order to obtain a greater effect on immune responses is now quite a common practice, especially in organ transplantation, although the regimes are generally arrived at by trial and error and with little certain knowledge of the basis of these synergistic effects. Dukor & Dietrich (1970) have shown that cortisone and cyclophosphamide are very effective as immunosuppressants when the antigen is preceded by the steroid and followed by the cytostatic drug. They suggest that the cortisone interferes with agent handling and the cyclophosphamide prevents the production of specific clones of immune cells. Makinodan et. al. (1970) have further shown that this type of regime is useful for inducing 'tolerance' to an antigen which is given i.v. between the two drug treatments. The mechanism here probably involves a permanent destruction of the specific stem cells by the alkylating agent as they are engaged in clone formation.

It has been demonstrated in this SECTION that PA alone, if given in an extended course of injections can interfere with the normal pathogenesis of scrapie agents in the periphery. Attempts to

use cyclophosphamide alone, however, were not successful (Table 4.1), as has also been demonstrated by other workers (see especially Worthington & Clark, 1971). It seemed, however, reasonable to try a combined treatment with the two drugs to find whether they would act synergistically to destroy the host's capacity to support scrapie pathogenesis in peripheral tissues.

Adult VL mice were treated s.c. with 500 mg/kg dose of PA in Tween 20/saline and two days later with 100 mg/kg dose i.p. of cyclophosphamide (CPA) in saline. Controls were given equivalent vehicle injections. Six days later all mice received i.p. injections of standard 79A supernate.

Incubation periods were as follows:

Table 4.15

Group	Incubation period	P
Controls	205 ± 3	
PA/CPA-treated	227 ± 3	0.001

This increase in incubation period, without a concomitant increase in variability, was the result of just two drug injections in adult mice given about one week before scrapie. Taken with the results of the standard PA/Tween 20 treatment it suggests that there is a particular population of cells in the mouse, required for scrapie pathogenesis, which is destroyed by large doses of PA and whose replacement can be arrested by either a further course of PA injections or a single injection of an alkylating agent.

An attempt has been made to increase the above effect by adding a normal PA maintenance course to it. However, all the mice in the experiment died within two months of a wasting disease presumably due to excessive damage to lymphoidal tissues (Pierpaoli & Sorkin, 1972).

Ex. 4.13 An attempt to increase the effect of PA on the incubation period of 79A agent in VL mice by the addition of peritoneal cell provocation

One of the possible mechanisms of the PA-effect is to cause the immobilization of peritoneal macrophages, together with the scrapie inoculum, in the peritoneal cavity, thus effectively delaying the access by the agent to its replication sites in the lymphoreticular organs. Because glucocorticosteroids both immobilise phagocytes and severely reduce the numbers of circulating lymphocytes the barrier to agent transport by either or both of these types of leucocytes could be effective for several days (Thompson & van Furth, 1970). Such a delay may also permit time for the complete destruction of the agent by the phagocytes or other means of disposal discussed in SECTION 3. Analogies for these types of relation between leucocyte and antigen abound in the immunological literature (see SECTION 4 DISCUSSION).

If this model represents at least a part of the PA-effect (and one cannot exclude direct action by the steroid on the cells which support agent replication) then the prolongation of incubation periods could possibly be augmented if the number of peritoneal macrophages was increased by conventional provocation methods.

Adult VL mice were injected 6 days before scrapie with 3 ml. of sterile thioglycollate (TG) medium i.p. in order to increase the numbers of peritoneal macrophages (Argyris, 1968). Two days later (i.e. 4 days before scrapie) they received a s.c. dose of 250 mg/kg PA in Tween 20/saline. Three control groups received treatments as follows: PA only (no TG); Tween 20 vehicle only (no TG); and no pretreatment. All mice were injected at the same time with a standard 79A supernate i.p. The PA-treated mice then received a maintenance course of 12 s.c. injections of PA (25 mg/kg in Tween 20/saline) between 1 and 29 days after the scrapie injection. Incubation periods were as follows:

Table 4.16

Group		Incubation period	P (of difference from Tween 20 controls)
Untreated controls	females	-	
	males	203 ± 2	
Tween 20 controls	females	197 ± 4	
	males	206 ± 3	
PA-treated controls	females	221 ± 4	<0.01
	males	236 ± 5	<0.001
TG + PA treated	(Two male cases only at 215 & 260 days; 2 female and 1 male survivors at more than 400 days and*7 very early deaths)		

* All 7 died with kidney damage, 35 to 102 days after scrapie injection.

(On the basis of a repeat experiment at present in progress it has been shown that the kidney condition is caused by the drug treatment and not scrapie). Despite the loss of numbers in the

TG + PA group, however, the occurrence of three survivors beyond 400 days after injection supports the expectation that there is a synergistic effect between the two drug effects.

Also of interest is the 25 day longer incubation period in the PA controls than in the Tween 20 controls (sexes combined: $P = <0.001$). A tentative conclusion, because these results are so similar to the ones obtained in Ex. 4.12, is that the single CPA injection in that experiment was the equivalent of the 12 x 25 mg/kg maintenance injections of PA in this one. These PA control incubation periods also provide a good instance of the PA effect with 79A uncomplicated by age and oil effects. They are comparable to the results obtained in Ex. 4.10 with the ME7 and 22C agents in VL mice.

PILOT EXPERIMENTS WITH TWO OTHER DRUGS KNOWN TO AFFECT THE LYMPHORETICULAR SYSTEM

The effects upon the incubation periods of peripherally injected scrapie agents of factors like the age of the host, treatments with prednisone acetate, cyclophosphamide, arachis oil and thioglycollate, all seem to point to explanations in terms of natural or artificial upsets in the functions of the lymphoreticular system. In consequence, two other drugs known to influence lymphoreticular function have been tested at the pilot level: phytohaemagglutinin (PHA) and 6-hydroxydopamine.

Phytohaemagglutinin This is one of many plant lectins (Sharon & Lis, 1972) some of which have marked stimulatory effects upon various classes of leucocytes (Astaldi & Airo, 1969), resulting also in splenomegaly due to increase in white pulp (Gamble, 1966). The effects of the drug on immunological responses seem to be dependent upon the route of administration (Petrányi et. al., 1968) and these differences are perhaps resolvable in terms of work by Elves (1967) in which he showed that doses of PHA prior to antigen suppress immune responses while ones given afterwards enhance them. He suggested that prior treatments siphon off relevant cells or limited metabolites.

The two pilot experiments to follow are part of a wider project in this laboratory and their tentative observations are supported by the results obtained by my colleagues to whom I am grateful for their permission to quote them here.

Ex. 4.14 The effect of oral doses of PHA on the incubation period of oral doses of 79A scrapie

Not much use has been made in this thesis of the oral route of infection. Previous experience with the ME7 agent shows that mice injected orally with standard 10^{-2} supernates show longer incubation periods than the same dose by the i.p. route but with nearly 100% incidences of disease. The intention in the following experiment was to try to simulate in the mouse what might possibly be a natural route of infection for slow-viral agents in sheep, mink and humans. A recent suggestion (Asquith, et. al., 1970) that a mitogen detected in samples of milk-derived lactoglobulin could have

an ultimate plant origin prompted the inclusion of oral PHA treatment in this experiment.

31 male and female BRVR mice were dosed orally (using a 1 ml. syringe with the needle protected by a short length of flexible tubing) with either a 0.04 ml. dose of 10^{-3} standard 79A supernate, or with 0.04 ml. volumes of a mixture of 79A supernate and a PHA suspension in 199 medium. The concentrations of agent were adjusted to give the same dose. The PHA (Burroughs Wellcome Reagent Grade) was a 10% suspension in 199 medium absorbed with C57 erythrocytes to remove the haemagglutinating activity. Of the 23 control animals, only 2 developed scrapie which was an unexpectedly low incidence on the basis of ME7 experience and illustrates the consequence of transferring assumptions regarding one agent to another. Out of the 8 animals which received PHA + 79A, however, there were 4 cases. These are small numbers but they suggest that, at least in the case of limiting doses of scrapie, stimulation of the lymphoreticular system with PHA may increase susceptibility.

Gertner et. al., (1969) have shown that when chronic PHA-treatments are added to immunosuppressive treatments with axothioprine and prednisone in skin allograft experiments in dogs that there is a marked synergism. Therefore an experiment was performed in which PHA treatments were added to a PA (in arachis oil) regime. The PHA injections were given on five days between the scrapie injection and the first of the follow-up doses of PA. The agent was standard 79A supernate i.p. in VL male mice. There were no synergistic effects, but the arachis oil and PHA-only control results were as follows.

Table 4.17

Group	Incubation period	P of difference from saline controls
Saline controls	197 ± 0	
Arachis oil (controls)	206 ± 2	0.01
PHA-only (controls)	188 ± 1	0.001

These results are also evidence for PHA encouraging the incubation of scrapie. Because of their direct relevance to the substance of this SECTION and in view of the paucity of these PHA results, permission to quote the following results from an, as yet, unfinished i.p. titration of the ME7 agent in C3H mice was given by Dr. A.G. Dickinson. The diluent used in this titration was either saline or PHA in saline and the following table shows the incidence of scrapie in the various titration groups by 360 days after injection:-

Incidence of scrapie to date

Titration group	Saline diluent	PHA diluent
10^{-2}	7/7	7/7
10^{-3}	6/8	8/8
10^{-4}	1/9	7/7
10^{-5}	1/10	2/9
10^{-6}	no cases yet	no cases yet

The 100% incidence in the 10^{-4} group treated with PHA as compared with 11% in the saline controls supports the effects described in my experiments above. Whether the results will finally show merely

a shortening of incubation period or an increase of effective titre remains to be seen.

In another experiment by Dr. Dickinson a 10^{-1} oral dose of ME7 in BALB mice was preceded 4 days earlier by a single i.p. injection of PHA, and 3 hrs. before with a single oral dose of PHA. The incubation periods of the controls and PHA-treated animals were 304 ± 9 and 336 ± 15 respectively - the latter group including a single early case of 253 days and two survivors of more than 500 days. This is possibly an example of prolonged incubation periods resulting from a prior treatment with PHA.

These results therefore all suggest that PHA-stimulation can profoundly influence the course of scrapie pathogenesis and that whether it encourages or discourages the disease could depend very much on the relative timing of the treatment with respect to infection.

6-Hydroxydopamine (6OHDA)

The second drug to be tested at the pilot level is 6-hydroxydopamine. When given neonatally this substance causes a drastic 'sympathectomy' and general permanent reduction in the levels of nor-epinephrine and dopamine in several tissues, including the brain (Angeletti, 1971). It has also been reported to upset cellular immune responses so that treated animals will accept skin homografts when adult (Stern, 1971).

Ex. 4.15 Two litters of VL mice were randomised to exclude any litter-bias. One litter was given four i.p. injections of 6OHDA

(150µg/g) in 0.02 ml. 2% ascorbic acid in saline on days 0, 2, 4 and 7 after birth. The control litter received the ascorbic acid antioxidant injections only. On the 29th day of life all mice were injected with a standard 79A supernate i.p. The incubation periods were as follows:

Table 4.18

Group	Incubation period
Ascorbic acid controls	206 ± 3
6OHDA-treated	213 ± 2 (9 cases and one 'survivor' after 500+ days)

The increased mean incubation period has a chance probability of only 10-20%, but the 'survivor' is remarkable. As has been amply illustrated in experiments using 79A agent in adult VL mice, survivors at this age and after standard doses are unknown except when large doses of steroid were given around the time of scrapie injection. This result suggests that a more refined dose regime may be possible using neonatal 6OHDA injection which could permanently protect mice against i.p. injections of scrapie. If this proves to be the case it will have then to be discovered whether the drug is having this effect by influencing peripheral or central systems.

A POSSIBLE PHARMACOLOGICAL MODIFICATION OF THE INCUBATION PERIOD OF CENTRALLY (I.C.) INJECTED SCRAPIE

All the experiments so far described in this SECTION investigated the effects of drugs upon the incubation period of scrapie

administered by a peripheral route (i.p. or oral). The following experiment shows an effect of Actinomycin D on the incubation period of i.c.-injected scrapie.

The titre of ME7 agent after i.c. injection in C57 mice begins to rise rapidly in the brain about 60 days later (Eklund, et. al., 1965; Dickinson, et. al., 1969). This may, of course, represent only the first readily detectable increase in titre, but there is accumulating evidence of a sudden onset of numerous changes in infected brains at this time, so it could be that agent which has merely been accumulating at critical sites within the brain up until 60 days suddenly begins to replicate with a consequent upset of normal function. If there is such a critical period then it could possibly be affected by injections of substances known to interfere with nucleic acid metabolism, even if only because of an impairement of host functions on which replication might depend. An increase in DNA metabolism has been demonstrated in mouse brains starting about 50 days after an i.c. injection of the "Chandler agent" (Kimberlin, 1972).

Ex. 4.15 Experiment to test the effect of injections of 6-mercaptopurine (6MP) and actinomycin D (AMD) on the incubation period of i.c. injected 79A

23 A2G mice (5-7 weeks old) were injected with a standard 79A supernate i.c. 10 of these were controls and were given no further treatment. From the 49th-81st days after the i.c. injection two kinds of drug treatments were given to experimental groups. 8 A2G mice received daily i.p. injections of 0.2 or 0.4 mg/mouse 6MP

(freshly prepared every 3 days in 0.1N NaOH and kept in the dark) from day 49-81.

Another 5 mice were given 2 µg/mouse doses i.p. of AMD using the following regime:

1st week: 7 daily doses;
2nd week: no injections;
3rd week: 6 daily injections;
4th week: no injections;
5th week: 3 doses.

This timing of the doses was determined by observation of the daily fluctuations of body weight. Four out of the five mice in this group showed such drastic reductions in body weight during the 5th week that they would probably have died if uninterrupted treatment had continued.

Incubation periods were as follows:

Table 4.19

Group	Incubation period	P
Controls (untreated)	167 ± 2	
6MP (0.2-0.4 mg/mouse/day)	168 ± 2	>0.1
AMD	181 ± 3	0.01-0.001

6MP is a base analogue known to interfere with nucleic acid synthesis, but injections of it do not appear to affect scrapie incubation periods (See also Table 4.1).

AMD, on the other hand, appears to have increased the incubation period of this i.c. injected agent by a significant amount although it did not have any effect at roughly comparable dosage after an i.p. injection (Table 4.1). It is worth noting here that the appearance of vacuolar lesions and the onset of drinking and feeding changes in A2G mice after i.c. injections of ME7 all occur during the period of treatment which was used in this experiment (SECTION 6 and APPENDIX 4).

SECTION 4: DISCUSSION: THE PHARMACOLOGICAL MODIFICATION OF THE INCUBATION PERIODS OF SCRAPIE AGENTS

Although most of this section has been devoted to a description of various more or less successful attempts to modify incubation periods these must not be allowed to obscure the fact that the most obvious feature of all attempts to do this is their great difficulty. In fact no successful pharmacological modification of scrapie incubation periods has previously been published. Most other successful methods have changed incubation periods at the cost of some obvious damage to the host. Measures such as surgical and genetical splenectomy (Fraser & Dickinson, 1970; Dickinson & Fraser, 1972) seem to have worked by depriving the agent of at least some of the sites of peripheral replication. It is, of course possible, that the PA-effect is also a kind of temporary 'chemical splenectomy' and so comes under this category, as may also be the case with AMD. But a direct attempt to induce chemical splenectomy with ethyl palmitate did not influence incubation periods (Fraser, pers. comm.).

The difficulty experienced in changing scrapie incubation periods using pharmacological means may simply be due to a failure to find the appropriate drug, but perhaps, in view of what we already know about this agent, it is more likely to be because the agent's replication is so closely bound up with some basic host function that it can only be affected by treatments which will also harm the host. The very regularity of the incubation period of these agents, which it is our intention to upset, is itself an indication that they are probably intimately involved with some homeostatically controlled process of the mouse.

The only methods of significantly changing scrapie incubation periods without surgical damage to the host that have been published so far are those reported by Dickinson, Fraser, Meikle and Outram (1972) in which a prior injection of an agent with a 'long' incubation period in a particular genotype of host prolongs the incubation period of a subsequently injected 'short' incubation period agent. The most probable mechanism for this effect is considered to be one of site-competition. This is rather a case of setting a thief to catch a thief, and it may be that in the last analysis only very scrapie-like molecules will be able to produce fairly specific rather than crudely non-specific interference with scrapie replication and do so without also damaging the host in some way. It has often been suggested (e.g. Gardiner & Marucci, 1969) that the lack of immunogenicity of scrapie agents may be due to their resemblance to some host antigens, and it could be that there are normal replicating molecules which function in host control mechanisms, with which scrapie agents are somehow able to compete

(See Final Discussion SECTION 7).

Another feature of nearly all these successful treatments is that they generally increase incubation periods. The only way of shortening incubation periods for which there is any experimental evidence, is the use of PHA after low effective titres of agent.

Prednisone acetate

The pharmacological treatment which has been explored the most in this thesis is that of massive doses of prednisone acetate, s.c. during the period immediately before and after an i.p. injection of scrapie agents. PA is an artificial steroid with enhanced glucocorticoid and reduced mineralocorticoid effects which was developed specifically for chronic administration where its anti-inflammatory effects were required without the sodium-retaining effects which are inevitably present with the natural cortisone.

An examination of the gross effects of single doses of 500 mg/kg s.c. in adult VL mice of various ages is reported in APPENDIX 2, where it is shown that all the expected effects of corticosteroid treatment on lymphoreticular organ weight and differential leucocyte counts were produced. Not only is there a dramatic reduction in organ weights but there is a marked change in differential leucocyte count including reduced proportions and absolute numbers of small lymphocytes and elevated numbers of polymorphonucleocytes.

It is generally considered that PA has about 3 times the glucocorticoid effect of cortisone acetate, so the effective levels

used on adults in these scrapie experiments greatly exceeds that generally used in experimental work. It is remarkable therefore that apart from a slight reduction in body weight during the period of treatment, the mice seem to remain quite healthy and to return to normal body weights soon after the last injection. Only when the drug was given to very young mice, or in a regime that also included other drug treatments like cyclophosphamide or thioglycollate have there been instances of persistent low weight, kidney-failure, and wasting disease.

The chief features of the PA-effect upon scrapie incubation periods are as follows:

- prolonged incubation periods are obtained, especially when high initial doses are used and there is at least three weeks maintenance treatment starting soon after the scrapie injection;

- it is easier to produce this effect in very young than in older mice, in the sense that lower and fewer doses are needed. The most effective point appears to be when the scrapie is given between one and two weeks after birth;

- it is not yet possible, however, to distinguish the contributions to this effect of age of host, PA- and arachis oil-treatments;

- PA injections given in early life do not have a permanent affect on the host's susceptibility to scrapie;

- some kinds of regimes, e.g. single injections of PA within the first few days of life, and perhaps spaced treatments later, may conduce to a shortening of incubation period, perhaps by producing an

increase in the scrapie-supportive tissues due to a rebound phenomenon.

These observations are not yet complete enough to more than indicate possible reasons for the PA-effect. Steroids influence so many aspects of the physiology of mammals that it is possible to suggest many models that are compatible with the data, and do so with the lurking suspicion that the relevant effect may be beyond the frontiers of present knowledge. However, from what is known of the association of scrapie with the lymphoreticular system, and of the effects of splenectomy and of hereditarily spleenless mice in increasing scrapie incubation periods, it is very likely that the cause of the steroid-effect is to be found somewhere in its well-known capacity for lymphoreticular depression. This discussion will therefore continue on this assumption and some other possibilities will be reserved for the final discussion in SECTION 7.

High doses of steroids (at least in rodents) have marked immunosuppressive effects both on the humoural and cellular fronts (Berglund, 1956, 1962; Kass, 1960; Cohen & Claman, 1971). These are perhaps due to the accompanying great reduction in circulating lymphocytes, (which is partly the result of lysis and partly to sequestration in unknown sites) and to the reduction of lymphoid areas in the spleen, lymph-nodes and thymus (Ishidate & Metcalf, 1963; Elliott & Sinclair, 1968; Cohen, Fischbach & Claman, 1970). Also of interest is the anti-inflammatory effect of many steroids which has been variously attributed to the stabilisation of lysosomal membranes (Bunim, 1959; Symons, et. al., 1969) and the immobilization of phagocytes in whatever tissue they happen to be found at the time

of treatment (Thompson & van Furth, 1970). Of particular note here, especially in view of the negative results in scrapie experiments reported from the use singly of drugs like cyclophosphamide, 6-mercaptopurine and actinomycin D (Table 4.1 and Worthington & Clark, 1971), is the demonstration that there are populations of cells in lymphoid tissues that show resistance to lysis by some of these substances but not to others (Miller & Cole, 1967). Evidence for a 'steroid-resistant' population of cells in the medullary cords of the thymus which is fully capable of mounting a graft-versus-host reaction has been put forward by Blomgren & Anderson (1969) and Cohen, Fischbach & Claman (1970). Similar populations of steroid-resistant cells, probably of thymic origin, have also been found in the spleen (Cohen, et. al., 1970) and in the bone-marrow (Levine & Claman, 1970) but these may have 'taken refuge' there as a result of the steroid treatment (Moorhead & Claman, 1972). It is likely that such cells, being members of the lymphoid population would succumb to steroid treatment at even higher doses such as the PA doses used in this thesis, and if so, these could include the scrapie-supportive cells that have been suggested on the basis of the neonatal age-effect (SECTION 3).

Of interest too, in view of the tentative results obtained with PHA is the fact that these steroid-resistant cells, in common with other θ-bearing cells are the bulk of the PHA-stimulable population of lymphoid tissues.

Another suggestive demonstration, by Nettesheim & Hammons (1970), showed that treatment of mice by several immunosuppressives

including 500 mg/kg of cortisone acetate interfered with antigen retention by the spleen (^{125}I human IgG). A similar failing could ultimately lie behind the finding (Ex. 4.2) of no scrapie agent in the spleen of a PA-treated survivor. These authors, however, also found this failure of antigen retention after 400r X-ray, actinomycin D (0.9 mg/kg), cyclophosphamide (300 mg/kg) (all single injections i.p. in saline). This suggests that even these treatments could be successful in modifying scrapie pathogenesis provided a sufficient dose rate could be found and maintained. On the other hand they may fail even at high dose due to the lack of some other ancillary effect like macrophage immobilisation which is possessed by the steroid.

Thompson & van Furth (1970) have investigated the effects of glucocorticosteroids on the kinetics of mononuclear phagocytes in adult Swiss mice. They showed that single large doses (in the region of 750 mg/kg of hydrocortisone) reduced circulating monocytes for up to 2 weeks (probably by sequestration in an unknown site) and kept the numbers of peritoneal macrophages unchanged (probably by immobilization). They also found a great reduction of the numbers of circulating and peritoneal lymphocytes but a rise after a few days in the numbers of polymorphonucleocytes (cf. APPENDIX 2). This result suggests the possibility that the PA-treatment of mice with scrapie may cause the trapping of the homogenate in the peritoneum with immobilised macrophages long enough for the activity of the agent to be reduced or destroyed. This assumes that these steroid-immobilised macrophages are still fully capable of enzymic degradation but this may not be so. Although

there is evidence that steroids do in fact impair these types of functions, perhaps by stabilizing lysosomal membranes (Symons, et. al., 1969; Weissmann, 1967; Ignarro & Colombo, 1972) there are some conflicting findings (Hirsch & Church, 1961; Allison & Adcock, 1965; Alexander et. al., 1968). Recently Chandra & Seth (1972) reported enhanced intracellular killing of Staphylococcus aureus by polymorphonucleocytes from patients receiving, prednisolone treatment for a nephrotic syndrome. The effect of PA-treatments on the ability of macrophages to digest bacteria, brain homogenates and other organic materials has not yet been investigated.

Arachis oil

The possibility that phagocytic activity may be important in the peripheral pathogenesis of scrapie is also suggested by the effect, discovered by accident, of arachis oil in prolonging incubation periods in experiments where it was used as a vehicle for steroids. The effect is known to occur with 79A in VL and A2G mice, and 22A in VM mice. A preliminary investigation has been made on the effect upon peritoneal cells of VL mice of 0.1 ml. arachis oil i.p. Peritoneal washouts with heparinized saline were examined daily for one week after the i.p. injection. The total cell-count was increased ten times within 24 hrs. of the injection and then slowly returned to normal during the week. No attempt was made to differentiate between cell types, which were in any case in such an activated state that identification would probably have been doubtful. For three days after the injection many oil droplets were surrounded by clusters of large cells (probably macrophages) and numerous granulated cells

containing oil droplets were observed. Within a few minutes of placing the samples on the counting chamber, cells began to adhere to the glass and commence spreading. This seemed to apply even to the smallest round cells, tentatively identified with small lymphocytes. These observations prompted a search in the literature, and two isolated observations of possible relevance have so far been found. Boyd et. al., (1959) reported that extracts of Arachis hypogaea contained a lectin that showed weak agglutinating properties with some human blood samples. The erythrocyte antigen was not identifiable with any known blood group antigen and was tentatively assigned the designation 'Gy'. Sugar-competition experiments suggested that the receptor had a galactose-glucose termination. Previous to this, Long & Martin (1956), investigating the claims for an antirheumatic activity of a particular drug, found this activity to reside in the arachis oil base. Purification yielded what seemed to be 10-14 carbon amine attached to phospholipids which had about 10^7 times the desensitizing ability of cortisone acetate when tested on the tuberculin reaction in guinea-pigs sensitised to B.C.G. Curiously, neither of these observations seems to have been pursued any further. While either or both of them could be relevant to the effect of arachis oil on scrapie incubation periods it is not possible to say what mechanism is involved. A prolonging of incubation periods of i.p. injected agents could, however, be produced by diametrically opposite effects namely, by 'blockade' or enhanced phagocytosis. While 'blockade' would suspend any phagocytic activities it could result in a mere postponement of the pathogenetic process. Alternatively, the stimulation of phagocytosis and digestion could

result in an effective reduction in titre and hence increased incubation periods. These alternatives could be examined in a preliminary way using conventional substrates to measure phagocytic activity. There is also some evidence for a synergism between PA treatment and the prior injection of the macrophage stimulant, thioglycollate (Ex. 4.13). Macrophages could, however, play a more positive role in the pathogenesis of scrapie than the above suggestions have implied, and it is possible to demonstrate some suggestive parallels between the role of macrophages in immune responses and those postulated for scrapie pathogenesis. Fundamental immunological research has shown that macrophage-processing of antigen is often essential for a proper immune response (especially with particulate antigens) (Fishman, 1961; Hulley, et. al., 1964; Mosier, 1967; Pierce & Benacerraf, 1969). Such processing also seems to enhance their immunogenicity (Mitchison, 1969), although whether this is due to a mere 'cleaning-up' process or to an adjuvant-like association of the antigen with a RNA moiety, or even to the production of some specific 'informational RNA' which then directs the immune response, has not yet been settled (Fishman & Adler, 1963, 1968; Fishman, et. al., 1963; Adler, et. al., 1960; Pribnow & Silverman, 1967; Bishop, et. al., 1967; Gottlieb & Straus, 1969; Fishman, 1970). Linked with this kind of work are the observations of physical associations between macrophages containing antigen and lymphocytes, with the formation of temporary uropods (McFarland, et. al., 1966) and macrophage-lymphocyte 'islands' (Pierce & Benacerraf, 1969; Bona, et. al., 1972). The effects of prolonged exposure of antigen to macrophages are relevant in this

context too. Although in such circumstances most of the antigen is quickly digested, a part of it can be retained for longer periods in the phagolysosomes (Unanue & Askonas, 1968a, 1968b; Kolsch & Mitchison, 1968) and on the surface of the cell (Unanue, et. al., 1969). However, very prolonged exposure of antigen to macrophages, especially when these have been stimulated, can result in reduced antibody production in experimental systems (Jennings & Oates, 1967; Perkins & Mackinnodan, 1965; Ehreneich & Cohn, 1969). Finally, while antigen in splenic lysosomes may retain its antigenicity for some time, this may be lost quickly in hepatic Kupffer cells (Franzl, 1962) and the titre of scrapie agent is reportedly low in liver (Eklund, et. al., 1967). All these kinds of immunological observations are subject to numerous operational qualifications regarding exact regimes, antigens, host strains, modes of administrations etc. which while they are the bane of the immunologist, they do provide scrapie workers with some suggestive models.

Clearly all these possibilities of 'conversations' between different members of the lympho-reticular population involving as they do the passing of residual materials, could provide an ideal mechanism for the transport and even the replication of certain infectious agents, although there is no direct experimental evidence for anything of the sort with scrapie.

A tentative model

The following is an attempt to hold together the separate effects on incubation period due to age, PA and arachis oil.

(a) i.p. injected scrapie supernates are first ingested by peritoneal phagocytes including macrophages. Much of the non-scrapie material and perhaps some of the agent is digested and the products excreted in the normal manner. Some of the scrapie agent (associated or not with some donor-tissue component; see SECTION 5) survives to be passed to peritoneal lymphocytes or be carried into the circulation while still in, or on, the macrophages (peritoneal macrophage turn-over in Swiss mice is about 40 days: Thompson & van Furth, 1970). The titre of agent that emerges from this phase (and the timing of the emergence) will depend upon factors like: the maturity of the phagocytes (i.e. their ability both to ingest and then digest organic materials, which is a function of the individual's age), the degree of activity of the macrophages (whether or not stimulated by substances like mineral oils, glycogen, allogeneic serum, thioglycollate, or blockaded by cholesterol, carbon, silica etc.), and the mobility of macrophages and lymphocytes (whether or not immobilised or lysed or just subdued by steroid injections).

(b) these macrophage-processed agents (or donor-agent complexes, SECTION 5) are then transported to the replication sites which probably occur somewhere in the tissues of the lymphoreticular system. This transportation could be done by macrophages in circulation as monocytes, or by lymphocytes to which the agent has been passed by macrophages in a manner similar to that for antigens and RNA-antigen complexes.

This phase of pathogenesis could be upset by treatments

which either lyse or cause the sequestration of the transporting cells such as would be the action of PA on both small lymphocytes and monocytes.

(c) on arrival at the peripheral target sites which are capable of supporting the replication of the agent, some at least of the agent particles may be passed onto these cells which could be plasma cells or the lymphocytes of the cellular immune system. Agent in excess of site-number could perhaps be taken up by other elements like reticular cells which are thought by some to function as an antigen reserve for the maintenance of immunological memory. Or these cells could be themselves the site of agent replication.

Accumulation of agent at these target sites could obviously be prevented by factors like: complete absence due to maturational deficiency of transporting and/or receptor cells, or the inactivation or depletion of the relevant cells by drugs like PA at high dose.

Finally as an alternative or supplementary consideration, it may be noted that there is evidence that treatment with corticosteroids can prolong the life of laboratory animals (Friedman, et. al., 1965; Bellamy, 1968). It has often been suggested that some aspects of the pathology of scrapie resemble premature ageing. If scrapie produces something like a hastened ageing of a particular brain-cell population then the PA-treatment may prevent this occurring so rapidly. Other possible models will be discussed in SECTION 7.

Phytohaemagglutinin

My results reported in this SECTION with this drug must be regarded as tentative although they are supported by other evidence from the unit. In view of the possible light which these could throw on the PA- and neonatal age-effects it is worth discussing them briefly at this point.

PHA appears to stimulate the θ-bearing, thymus-derived lymphocytes, causing them to enter blastogenesis. In this state they have greatly enhanced capacities for supporting some viral infections in vitro (Herpes simplex: Nahmias, et. al., 1964; vesicular stomatitis: Edelman & Wheelock, 1966; mumps: Duc-Nguyen & Henle, 1966; vaccinia: Miller & Enders, 1968; yellow-fever: Wheelock & Edelman, 1969). Morphologically, PHA-treated mice show a splenomegaly with greatly increased quantities of white-pulp (Gamble, 1966). The effects of PHA stimulation on immunological responses are somewhat contradictory in the literature, apparently depending on route of injection (Petrányi, et. al., 1968). Such results are perhaps resolvable in terms of an experiment by Elves (1967) in which he showed that the antibody response of rats to chicken red blood cells was suppressed by prior doses of PHA but unaffected or enhanced by subsequent ones. He suggested that prior treatment 'siphoned off' some cells which were relevant to the immune response.

These observations, taken with the results of the scrapie experiments using PHA, suggest that there may be a PHA-sensitive population of cells that is of central importance in scrapie replication. This could, indeed be the relatively steroid-resistant

group postulated earlier, which could nevertheless succumb to very high, persistent doses. Prior treatment with PHA could pre-empt these cells or some necessary physiological process so that they were no longer available for scrapie replication and so allowing the scrapie inactivation mechanisms full reign for a time and thus increasing incubation periods, or on the other hand, simultaneous or subsequent PHA treatments (with respect to scrapie infection) may stimulate cells that are already infected, which could hasten the replication of the contained agent. This would shorten incubation periods of those doses of agent which do not already exceed the number to saturate available replication sites i.e. low titres. Hastened agent replication could be due in stimulated cells to either a generally more metabolically active environment or specifically to more rapid turnover of DNA which would accord with the suggestion that agent replication in tissue-culture is in concert with cell-division (Clarke & Haig, 1970).

If the postulated, limited number of sites (or cells) capable of supporting scrapie replication is a thymus derived, relatively steroid-resistant, PHA-stimulable one, then this may account for another slightly anomalous scrapie observation: that although the titre of agent after s.c. injections rises quickly in the spleen and lymph nodes, a rise in titre in the thymus is not detected until two months later - about the same time as the salivary gland. Of course, there are no germinal centres in the thymus and so on an analogy with antigen one would not expect agent to appear in this gland. However, it is very rich in lymphocytes, so it is possible that the infected lymphocytes in the gland are

simply a very small minority of the total population, such as the steroid-resistant cells. A similar explanation for the salivary gland would point to the small proportion of cells that are concerned with IgA production which are presumably the most likely candidates for the sites of scrapie infection. Another lymphoid organ which shows low agent titre is the ileum. Again the question of proportion of cells arises since the Peyer's patches are only a part of this organ, but it should be noted that θ-bearing cells seem to be absent from the lymphocytes of this tissue which is one reason for regarding it as the Bursa of Fabricius-equivalent in mammals and therefore composed entirely of B-cells (Heim, et. al., 1972).

However, these speculations regarding a possible role for T-cells (or at any rate θ-bearing cells) in scrapie pathogenesis have to be seen in the light of the demonstration by McFarlin, et. al., (1972) that adult thymectomy followed by irradiation and reconstitution with foetal liver cells did not significantly influence the incubation period of peripherally injected scrapie (although it was profound enough to cause a permanent loss of the ability to reject skin allografts and the complete disappearance of detectable θ-bearing lymphocytes in the spleen). However, this result does not necessarily discount the possibility of the kind of model I have proposed since the particular population of T-cells needed for scrapie replication may be widely dispersed in the adult mouse and also radiation-resistant, or alternatively, it (unlike the cells needed for allograft rejection) may have precursors in foetal liver cells.

6-hydroxydopamine

The result with this drug are even more preliminary than those with PHA so they will be discussed very briefly. 6OHDA causes a profound depression of catecholamine levels in several organs of the body, including the brain (Uretsky & Simmonds, 1971). Given neonatally it causes a permanent 'chemical sympathectomy' (Angeletti, 1971) and permits treated mice to accept skin allografts in adulthood (Stern, 1971). The effects on catecholamine levels are perhaps more relevant to the discussion of the changes in behaviour with scrapie which are dealt with in SECTION 6. The effect on cellular immune responses appears not yet to have any known basis. Devoino and Yeliseyeva (1971) have shown that another biogenic amine, 5-hydroxytryptamine, is involved in the immune response. Neonatal injections of epinephrine, especially when given with propiomazine HCl impairs both humoural and cellular immune responses in rabbits when adult (Henson, Ball & Brunson, 1968; Henson & Brunson, 1969). These results suggest that 6OHDA could also have direct effects on some cells of the immune system which may also be those required for scrapie pathogenesis.

Actinomycin D

With these results we pass to an entirely different aspect of scrapie pathogenesis. The AMD-effect was produced with i.c. injected scrapie. Reasons have been given in SECTION 1 for supposing that i.c. injected agents proceed to replicate <u>in situ</u> thus omitting several pathogenic stages which they would have been obliged to perform had they been injected by a peripheral route.

Kimberlin & Anger (1969) and Kimberlin & Hunter (1967) have demonstrated a brief period of ^{3}H-thymidine incorporation by sub-ependymal cells during the period immediately after an i.c. injection. Perhaps these are the first cells to be affected by the agent which show an immediate, transitory response in terms of DNA turnover which does not become widespread in the brain until several weeks later when another phase of DNA turnover begins (Kimberlin, 1972). Perhaps this occurs when the level of agent crosses the threshold for making this response in other parts of the brain as a result of accumulation and replication. This phase also includes the appearance of numerous other signs of malfunction: subtle behavioural changes (SECTION 6), vacuolation of neurones, astrocyte hypertrophy, rise in levels of some brain glycosidases, increased acetylation of histones (reviewed by Kimberlin, 1973) and a sharp decline in spermine levels (spermidine levels unchanged) (Shaw, pers. comm.). These changes constitute a great stepping up of activity which could represent the crossing of some metabolic threshold such that homeostasis can only be maintained by greater activity. AMD, which causes a breakdown of normal DNA metabolism (e.g. Wells, 1969; Brachet & Hulin, 1969) and is widely used for stopping the production of proteins (e.g. Honigg & Rabinovitz, 1965; Pastan & Friedman, 1968) could postpone scrapie incubation periods if injected at this critical point in pathogenesis, especially if scrapie replication requires normal host responses such as transcription. Similar to the lack of effect with 6MP is failure to change incubation periods with 5-iododeoxyuridine and monohydroxyurea reported by Kimberlin and Hunter (1967).

SECTION 5

EFFECTS ON THE PATHOGENESIS OF ME7 SCRAPIE WHICH MAY BE ATTRIBUTABLE TO DONOR TISSUE COMPONENTS

EFFECTS WHICH MAY BE ATTRIBUTABLE TO DONOR TISSUE COMPONENTS, ON THE PATHOGENESIS OF PERIPHERALLY INJECTED ME7 SCRAPIE

This SECTION is an account of my own contribution to a project in progress in these laboratories which has been designed to investigate another possible source of variation in scrapie pathogenesis, namely the potential effects of donor tissue components which are of necessity present in the inocula, if not actually complexed with the agents. The first results of this work have been published (Dickinson & Outram, 1973; Outram, Fraser & Wilson, 1973 - see APPENDIX 6). The following account will therefore begin with a brief synopsis of the project so far, and then go on to describe my own contributions and extensions.

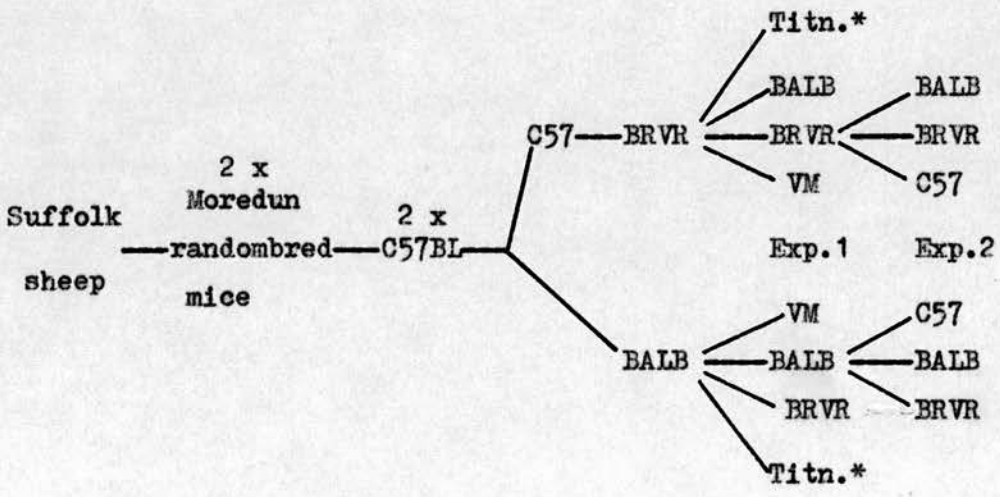
Summary of the design and results of the first phase of the BALB/BRVR project

In the course of the study of variation in experimental scrapie in mice it had been shown that the following factors were operative in terms of modifying incubation periods and lesion profiles: the strain of agent, the genotype of the host, the route of injection, the tissue-source (brain or spleen) and sometimes the sex of the recipient (See SECTION 2). The donor-tissue components which are inevitably present in the inocula are another potential source of variation. In order to study this possibility, however, it is necessary to use an agent source for which there is good, widely based evidence that it is stable in its properties and likely to be a single strain of agent rather than a mixture of strains. The ME7

agent meets these criteria better than any other which has been studied in detail. At the start of this project, ME7 had already been passaged by the i.c. or i.p. routes serially in several strains of mice without any signs of breaking down into separate components or of changing its properties in any other way, and in this it can be contrasted with the majority of isolates from sheep (Dickinson, pers. comm). ME7 has now retained its status in these respects during 11 years of intensive study in these laboratories. In particular, it has been passed 11 times in C57 mice (s7s7) and 7 times in VM mice (p7p7) and remained unchanged in its properties as indicated by incubation periods and lesion profiles in both these genotypes (Dickinson, 1970; Fraser & Dickinson, 1973; Dickinson, pers. comm.).

For the purpose of this investigation therefore, some ME7 material from 2nd C57 pass was injected into BALB, BRVR and further C57 mice using both the i.c. and the i.p. routes of injection in a

Passage history of material used in BALB/BRVR experiments
(from Dickinson & Outram, 1973)



* in both BALB abd BRVR recipients (See Table 5.1)

sequence of experiments which principally used changes in the P/C ratio (ratio of i.p. to i.c. incubation periods: see SECTION 2) as an indicator of changes in pathogenesis. In the event the passage of ME7 in either the BALB or the BRVR mice not only resulted in a marked difference between the two in P/C ratios but produced significant histological differences also. These are important for the interpretation of the causes of the modified patterns of pathogenesis. At the present time, the discovered changes are being investigated by passing the material back and forth between genotypes in order to determine what proportion of the differences between the BALB and BRVR sublines of ME7 are transitory properties due to the genotype of each inoculum - perhaps due to only the normal tissue antigens present.

An outline of the experimental procedures is as follows: standard ME7 supernates from the brains and spleens of terminally affected individual BALB and BRVR mice were injected by either the i.c. or the i.p. routes into weanling BALB and BRVR mice. Incubation periods and lesion profiles were obtained in the normal manner. Then BALB and BRVR brain and spleen materials from this experiment were used to inject a second set of BALB, BRVR and C57 mice by both the i.c. and the i.p. routes. The incubation periods from both these experiments are shown in Table 5.2. The lesion profile data was analysed using a multivariate technique specially developed for the purpose by Mr. D.T. Wilson and the results are illustrated in Figs.5.1-4 and described in full in the appended paper (Outram, Fraser & Wilson). Meanwhile cross-titrations by the i.c. route were carried out in BALB and BRVR mice of the BALB and BRVR materials used for the

first experiment, shown in the passage diagram. These showed that the titres were essentially the same in the two sources:

Table 5.1 Estimated titres (Reed-Muench)

Donor strain	Recipient strain	
	BALB	BRVR
BALB	6.5	6.6
BRVR	7.0	6.6

In addition both subline standard brain inocula were injected into VM (p7p7) mice because this has proved to provide the most sensitive indicator of agent/strain differences, but this gave no evidence that the two sublines were different.

The results of these two experiments in terms of incubation periods, P/C ratios and lesion profiles finally break down into 80 groups, and it is unnecessary to go into the details here. The chief points to emerge are that the i.c. data, so far obtained, all strongly suggest that the ME7 is essentially unchanged in its properties after passage in these diverse hosts but that some association with components of BRVR tissues results in changes in peripheral pathogenesis and subsequent central pathogenesis. These changes are expressable as a decreased neuroinvasiveness (larger P/C ratios) and markedly different lesion profiles of the BRVR-passed ME7 after i.p. injection in both strains of recipient, which could indicate a different pattern of agent distribution in the brain. This latter possibility cannot be established at this time since the relationship between lesion density and agent distribution is not known,

Table 5.2 Incubation periods for ME7 agent which had been passaged in either BALB or BRVR mice, and ratio of incubation periods by i.p. or i.c. routes (P/C ratio) in three recipient strains of mice

Donor strain	Organ & expt.	Recipient strain: BALB i.c.	BALB i.p.	BALB P/C	BRVR i.c.	BRVR i.p.	BRVR P/C	C57 i.c.	C57 i.p.	C57 P/C
BALB	Brain 1	178±1	271±3	1.52	170±1	243±1	1.43	-	-	-
	2	190±3	280±2	1.47	180±2	240±3	1.33	181±7	253±7	1.40
	Spleen 1	209±2	312±8	1.49	210±5	299±20	1.42	-	-	-
	2	231±6	341±29	1.58	202±3	258±7	1.28	208±3	339±44	1.63
BRVR	Brain 1	169±2	309±7	1.83	161±1	288±4	1.79	-	-	-
	2	174±2	302±7	1.74	166±1	275±5	1.66	176±14	278±1	1.58
	Spleen 1	218±1	353±5	1.62	195±2	329±7	1.69	-	-	-
	2	225±9	368±6	1.63	233±7	342±3	1.47	211±6	380±40	1.80

(From Dickinson & Outram, 1973)

EFFECT OF DONOR STRAIN ON LESION PROFILES

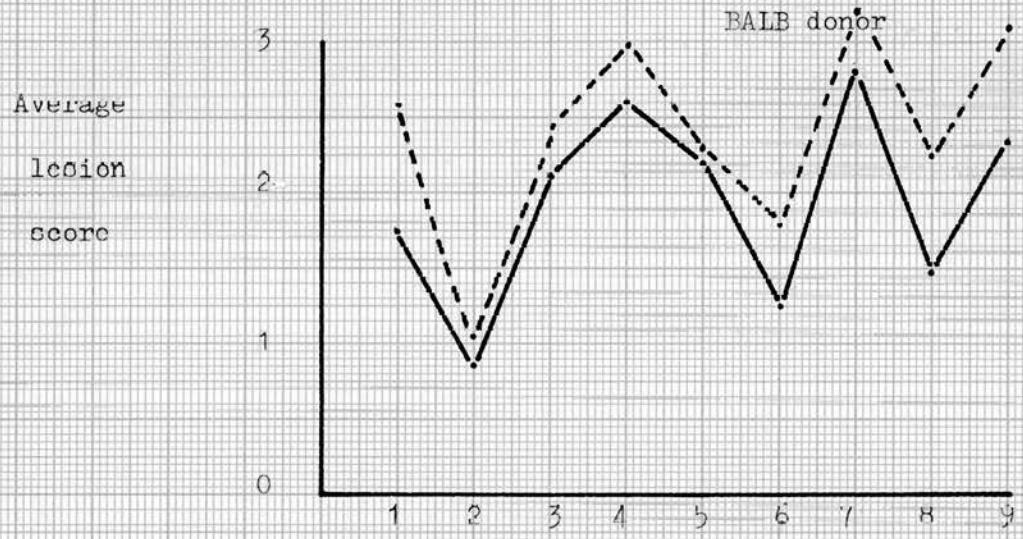


Figure 5.1 Lesion profiles of BALB passed ME7 in BALB recipients ——— and BRVR recipients - - - -

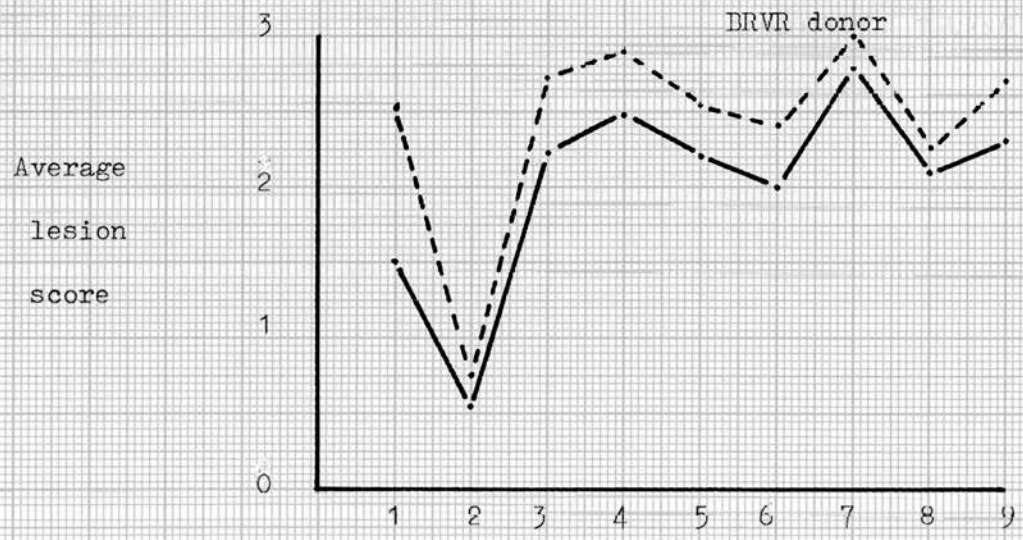


Figure 5.2 Lesion profiles of BRVR passed ME7 in BALB recipients ——— and BRVR recipients - - - -

EFFECT OF DONOR STRAIN ON LESION PROFILES

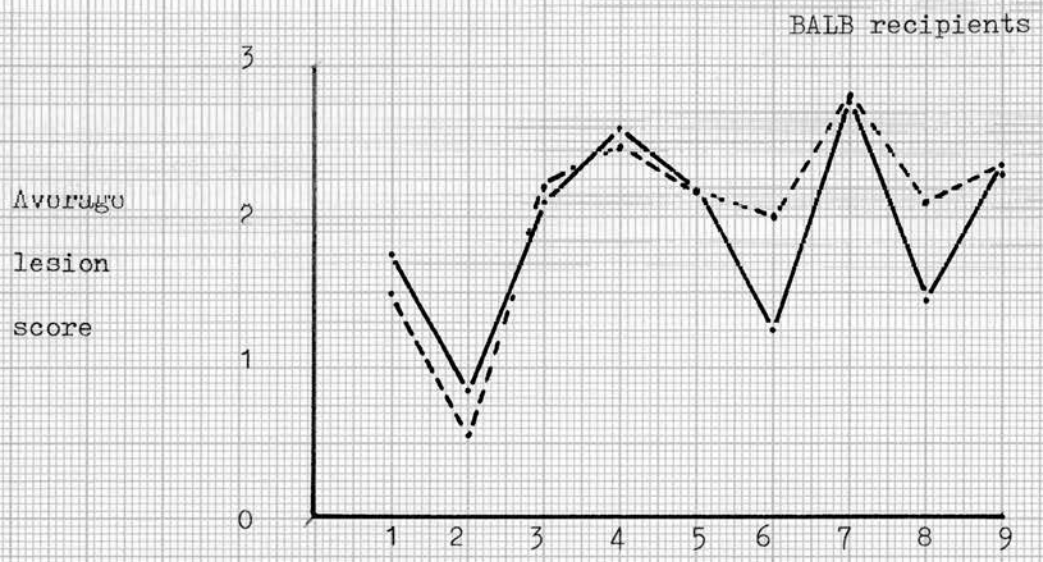


Figure 5.3 Lesion profiles in BALB recipients of ME7 (brain) injected i.p. from BALB ——— or BRVR - - - - donors

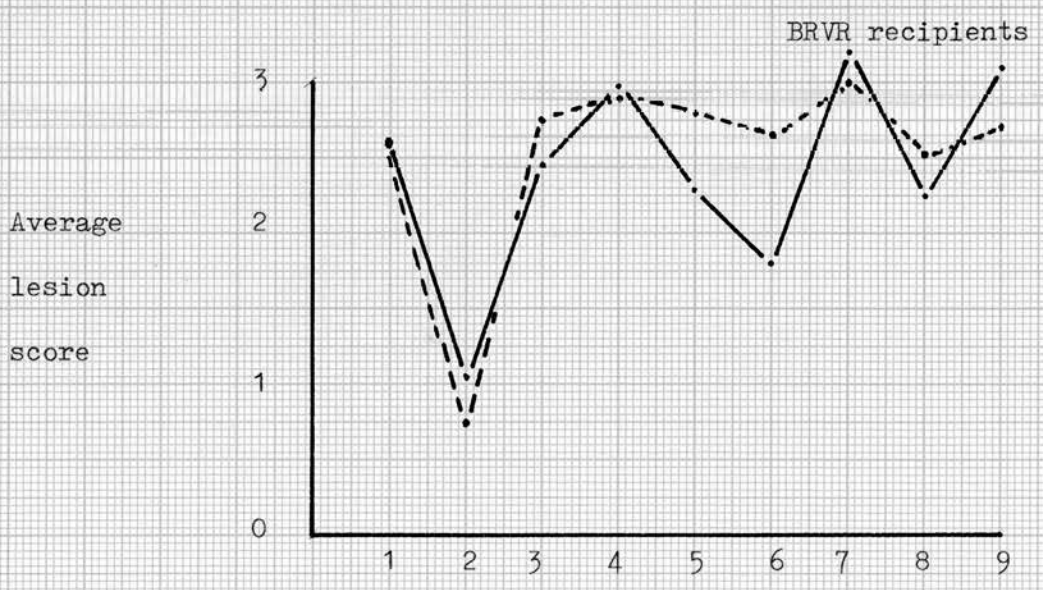


Figure 5.4 Lesion profiles in BRVR recipients of ME7 (brain) injected i.p. from BALB ——— or BRVR - - - - donors

but some general relationship of the two is not an unreasonable expectation.

Multivariate analysis of the lesion profile data showed that significant variations due to the strain of the recipient after i.p. injection occurred in positions 2,3,6,8 and 9, the most significant being at 6 (hippocampus) (Outram, Fraser & Wilson, 1973). The differences due to donor strain in individual brain positions after the i.c. route were even more significant than those after the i.p. route but an analysis of the change of shape showed that the cause of these larger variations was largely one of level of intensity rather than difference in shape.

If the patterns of lesion distribution reflect in some degree the distribution of agent then it is fairly easy to envisage simple physico-chemical causes for differential distribution of the inoculum after an i.c. injection, according to the strain of the donor. Presumably even normal tissue components from different genotypes could be differently distributed. These differentials could bias the sites where agent replication would begin. It is more difficult, however, to see how donor tissue components could influence lesion profiles after a peripheral injection, especially since the agent does not appear to gain access to the brain until several weeks after injection. The possible mechanisms include: some kind of 'priming' of the brain by the inoculum at the time of injection which then influences the pattern of subsequent agent damage; infection of the brain could be at least partly by unchanged homogenate (i.e. still associated with donor components) whether immediately or after several weeks of storage

in some peripheral tissue; the replication of donor-tissue molecules along with the agent in peripheral cells - (even this type of possibility cannot be entirely dismissed); or the enlisting of host specific immune responses to the donor components, leading to some incipient autoimmune reaction which results in a deposition of the agent in corresponding target sites ('immunological aiming', see later).

My contribution to this project

I was given the opportunity to examine the histological data of the first set of the BALB/BRVR experiments and to start some experiments to test for the possibility that immunological mechanisms were involved in the changed P/C ratios and lesion profiles.

Results

The analysis of the histological data led to two findings. One (described above) was that the shape of the lesion profile in i.p. injected animals was, visually, more obviously influenced by the strain of the donor than by that of the recipient. This was the first time that an effect on lesion profiles due to the strain of the donor had been observed in these laboratories. Subsequent multivariate analysis, however, showed that recipient effects were in fact as significant though less obvious to the eye and to simpler forms of analysis. (Fraser, 1971; Outram, Fraser & Wilson, 1973).

The second histological observation was the discovery that a high incidence of white-matter oedema was commoner in animals receiving BRVR-passed ME7 (brain or spleen) than in animals receiving

BALB-passed ME7; this effect was most marked after i.p. injections. This association of white-matter oedema with BRVR-passed ME7 is another indication that a modified pathogenesis occurs in ME7 from this host genotype. The percentage incidence of this lesion and χ^2 tests of its association with donor and recipient genotypes are shown in Tables 5.3 and 5.4.

Table 5.3

Donor	Recipient	% of mice showing white-matter oedema			
		i.c.		i.p.	
		n	%	n	%
BALB	BALB	63	68	40	15
	BRVR	26	35	31	29
BRVR	BALB	43	70	39	90
	BRVR	45	60	49	86

Table 5.4 χ^2 tests of the significance of the associations of the % white matter oedema incidences with the two donors and recipients

Donors	Recipients	P	
		i.c.	i.p.
BALB & BRVR	BALB	>0.1	<0.001
"	BRVR	.05-.02	<0.001
BALB	BALB & BRVR	.01-.001	>0.1
BRVR	"	>0.1	>0.1

Ex. 5.1 An experiment to investigate the possibility that normal BRVR brain may contain a factor which could modify the pathogenesis of BALB-passed ME7

38 BALB mice were injected by the i.p. route with a standard brain supernate of BALB-passed ME7 to which had been added an equal volume of normal brain supernate taken from either a BALB or a BRVR mouse. The object was to look for any changes of incubation period, lesion profile or white-matter oedema due to the BRVR normal brain.

The results are shown in Table 5.5 and Fig. 5.5

Table 5.5

	Incubation period	White-matter oedema
BALB-passed ME7 + BALB brain	257 ± 2	nil
BALB-passed ME7 + BRVR brain	258 ± 2	nil

The absence of significant differences in the pathogenesis of the BALB-passed ME7 in the two inocula in terms of incubation period and lesion profile counts against the possibility that the BRVR stock contains an infectious entity which somehow influences the peripheral pathogenesis of scrapie. It suggests instead that in infected BRVR inocula there are components which are either absent from normal BRVR brain or present at much higher concentrations, or are complexed with the agent in a manner which a mere mixing of inocula does not simulate, and that these components are responsible for the changed pathogenesis.

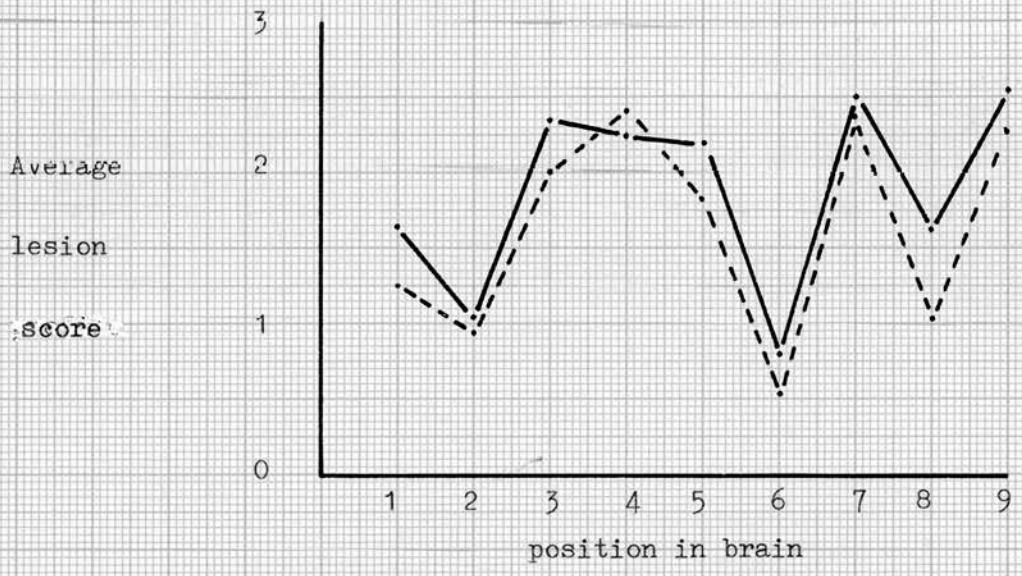


Figure 5.5 Lesion profiles of BALB passed ME7 (brain) i.p. in BALB recipients after the addition of normal BALB —— or BRVR - - - - brain to the inoculum.

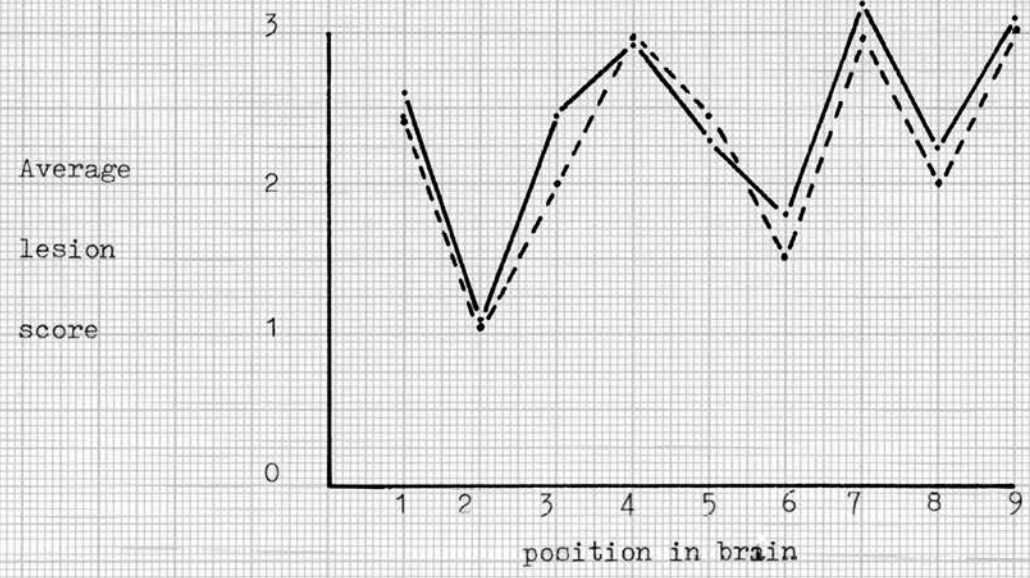


Figure 5.6 Lesion profiles of BALB passed ME7 (brain) i.p. in BRVR recipients in the original BALB/BRVR experiments —— and in BRVR recipients pretreated with normal BALB brain i.p. - - - -

Ex. 5.2 An experiment to investigate the effect of several pretreatments of BALB or BRVR mice, respectively, with normal BRVR or BALB brain homogenate, before injecting ME7 in allogeneic brain by the i.p. route

This was another attempt to see if normal tissues contained factors which would influence the pathogenesis of i.p. injected scrapie. BALB and BRVR mice were given courses of their own (isogeneic) or each other's (allogeneic) brain suspensions i.p. for several weeks before receiving i.p. injections of allogeneic brain infected with ME7. It was expected that such pretreatments might exaggerate the changes in pathogenesis associated with allogeneic donors.

55 BALB and 42 BRVR mice were each divided into 5 groups as shown in Table 5.6. The group 5s were the basic controls receiving no form of pre-treatment before the i.p. injection of standard ME7 brain supernate (0.1 ml.) in allogeneic tissue. The group 4s constituted a second control receiving a full course of three-pretreatments with isogeneic normal brain before receiving ME7 from the isogeneic source. Groups 1-3 received up to 3 pretreatments i.p. with normal allogeneic brain before the i.p. injection with standard allogeneic ME7 brain supernate. (The ME7 materials were obtained from the first of the original BALB/BRVR experiments so that, except for the dose of agent, this experiment (5.2) represents the same passage status as the second of the original BALB/BRVR experiments).

A comparison of titre in the BALB- and BRVR-passed materials was made by i.c. injection of RIII mice. The incubation periods were 161 ± 8 days for the BALB material and 155 ± 3 days for the BRVR, indicating almost identical titres.

The incubation period results are shown in Table 5.7 and the lesion profiles in Figs. 5.6 and 5.7.

Table 5.6

Day of injection of normal brain supernate relative to scrapie	Dose of allogeneic pre-treatments and scrapie supernate				Dose of isogeneic pretreatments and scrapie supernate
Group:	1	2	3	5	4
- 29 days	10^{-3}				10^{-3}
- 21 days	10^{-2}	10^{-3}			10^{-2}
- 14 days	10^{-2}	10^{-2}	10^{-2}		10^{-2}
0 (Scrapie, 0.1 ml from allogeneic donor)	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2} (from isogeneic donor)

Table 5.7

Donors	Recipients	Group	Incubation period	Presence (+) or absence (-) of white matter oedema
BALB	BRVR	1	214 ± 2	-
		2	216 ± 2	-
		3	217 ± 2	-
		5	217 ± 3	-
BRVR		4	244 ± 11	+
BRVR	BALB	1	290 ± 6	+
		2	277 ± 9	+
		3	303 ± 8	+
		5	279 ± 9	+
BALB		4	260 ± 13	-

The incubation periods in the control groups (4 & 5) show the same kinds of relations to each other as the combinations in the original experiments, except that they are about 25 days earlier probably because of the greater volume (0.1 ml.) of inoculum used. It can be seen that the BRVR source of ME7, although having the slightly shorter incubation period in the RIII i.c. titre controls, showed a reduced neuroinvasiveness as compared with the BALB source material when injected i.p. and it also produced the white-matter oedema that is characteristic of BRVR-passed ME7.

The pretreatment of BRVR mice with normal BALB brain before injections with BALB-passed ME7 had no effect on pathogenesis either in terms of incubation periods or lesion profiles (Fig. 5.6). However, the pretreatment of BALB mice with normal BRVR brain produced marked changes in the mean incubation periods of the various groups, and the lesion profiles appear to have been modified also. There are significant variance ratios ($P < .05$) of group 4 compared with groups 1,2,3 & 5 in both halves of this experiment urging some caution in the direct comparison of mean differences by t-tests. When the incubation period results are classified by sex (Table 5.8) it can be seen that the pretreatments have marginally increased the incubation periods of the females but not of the males. The variance ratio for the treated and all the untreated females shows a significant change ($P .01$) in the distribution of incubation periods as a result of treatment.

Table 5.8

Donors	Recipients	Number of pre-treatments	Group	Incubation period and significance of difference from control mean			
				females	P	males	P
BRVR	BALB	3	1	305± 5	0.05-0.02	270± 8	0.1
		2	2	277±14	0.1	277± 8	0.1
		1	3	307±10	0.1-0.05	293± 8	0.1
		0	5	283± 8		271±24	

The original experiments showed that the change in neuroinvasiveness in the BRVR-passage line was accompanied by significant changes in lesion profile. This is illustrated clearly in Figs. 5.3 & 4. (See also Outram, Fraser & Wilson, 1973). In order to decide whether the lesion profiles were altered as a result of the pretreatments in Ex. 5.2 it was necessary, because of small numbers, to amalgamate the three pretreatment groups (1,2 and 3). The contemporary control groups were also too small to be the basis for a reliable comparison. Instead, a comparison was made of the lesion profile for all the BALB mice pretreated with BRVR brain in this experiment with that of the BALB mice injected with BRVR-passed ME7 in the original experiments (i.e. without any pre-treatments) and this shows some interesting features (Figs. 5.7 and 5.8). It can be seen that while pretreatment of BRVR mice with BALB normal tissues has not changed the lesion profile (Fig. 5.6), the pretreatment of BALB mice with BRVR normal tissue has. In particular, the scores in positions 5, 6 and 7 are higher than in the non-pretreated BALBs. If one compares the direction of the change of shape in lesion profile of BRVR-passed ME7

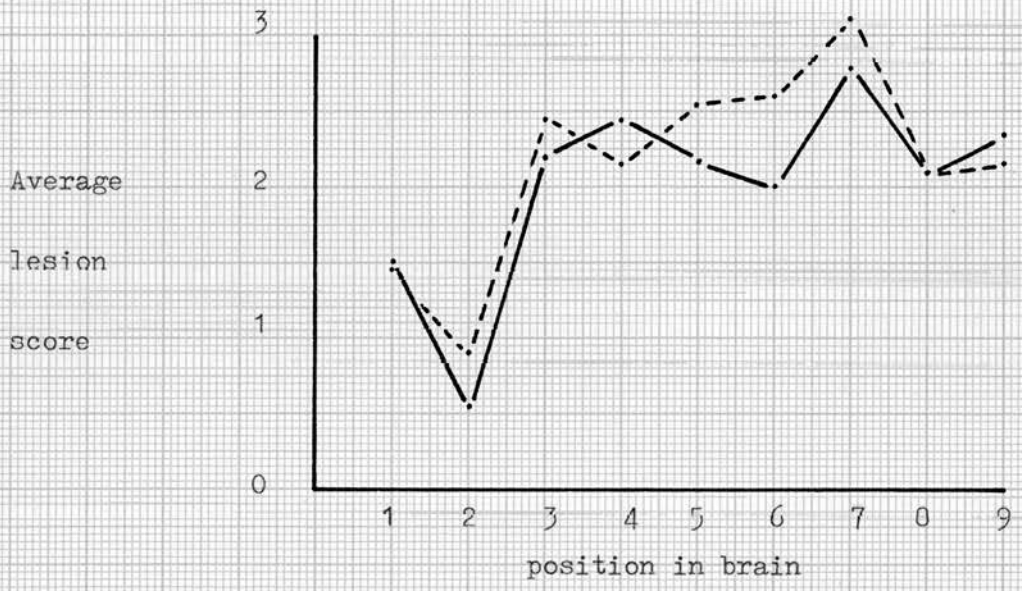


Figure 5.7 Lesion profiles of BRVR passed ME7 (brain) i.p. in BALB recipients in the original BALB/BRVR experiments ——— and in BALB recipients pretreated with normal BRVR brain i.p. - - - -

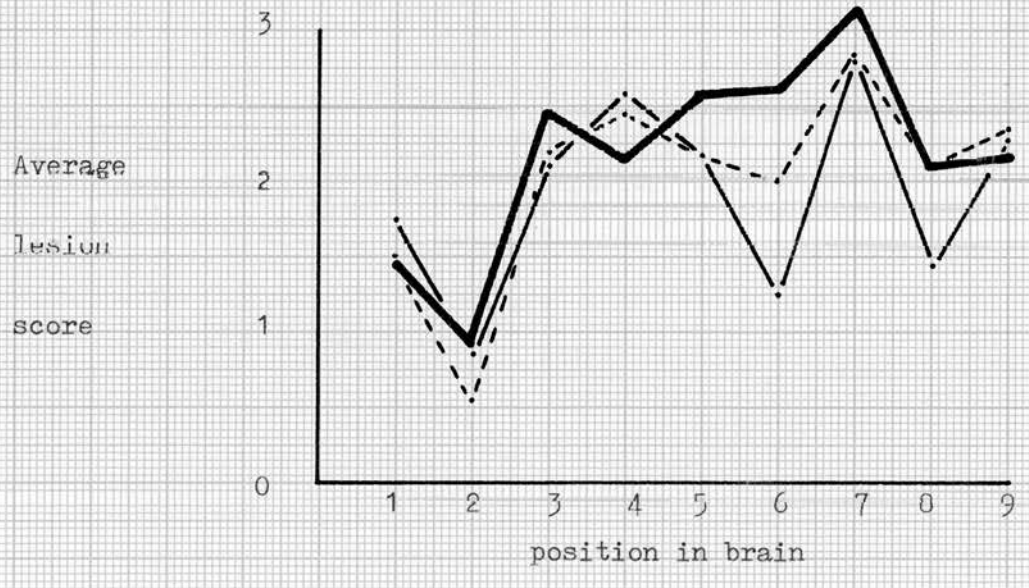


Figure 5.8 Lesion profile of BRVR passed ME7 (brain) i.p. in BALB recipients pretreated with normal tissues ——— superimposed on profiles of the BALB recipients in the original BALB/BRVR experiments (Fig. 5.3) and illustrating the amplification by BRVR pretreatment of the change of shape produced by BRVR passed ME7 - - - - when compared with BALB passed ME7 ———

as compared with that of BALB-passed ME7 (Figs. 5.1-4), then it can be seen that pre-treatment with BRVR normal tissue in this experiment has amplified some of these differences (See Fig. 5.8). A Lawley-Hotelling multivariate t-test was used to estimate the significance of this amplifying trend, and it was found to have a chance likelihood of only 1%. It is concluded that the pretreatment of BALB mice with normal BRVR brain material prior to injections of BRVR-passed ME7 has tended to decrease neuroinvasiveness still further and to exaggerate the changes in lesion profile shape.

SECTION 5 DISCUSSION: THE ROLE OF DONOR-TISSUE COMPONENTS IN THE PATHOGENESIS OF PERIPHERALLY INJECTED SCRAPIE

This subject is under active investigation in these laboratories at the present time and the data presented in this section can do no more than indicate that an effect of donor tissue components has been established in the case of one particular agent/host strain combination and to go some way towards suggesting possible ways in which they could be operating.

The general significance of the original BALB/BRVR experiments have already been discussed earlier in this SECTION, so it remains to consider in what ways the results of the two experiments reported in Ex. 5.1 and 5.2 can add to our understanding. They indicate that while the administration of normal BRVR brain material at the same time as an injection of BALB-passed ME7 i.p. in BALB mice has no effect on pathogenesis (Ex. 5.1), the pretreatment of BALB mice with normal BRVR brain two weeks or more prior to an injection of BRVR-passed ME7 does

influence pathogenesis (Ex. 5.2). The form which this influence takes is to increase the variance of the incubation periods with a slight increase of the mean and to continue further the trends in the changes of lesion profile shape which BRVR-passed ME7 shows compared with BALB-passed ME7. Additional experiments to check and extend these findings are in progress.

The effect of the BRVR tissue may be due to some generalised stimulation of the reticulo-endothelial system such as can be obtained with mineral oils, and thioglycollate medium and arachis oil, SECTION 4). If this prolongation was therefore due to agent inactivation this would show up as an even lower than usual estimate of titre by the i.p. route than by the i.c. one when BRVR donor material is used, but not when it is BALB material. Furthermore the estimates of titre would be even lower still if the assay animals had been pretreated with normal BRVR tissues.

While the incubation period changes can easily be thought of in terms of general reticulo-endothelial activation, the lesion profile changes cannot. As was mentioned earlier in this SECTION it is reasonable to suggest that a higher lesion score in a particular region of the brain is a reflection of higher agent activity there (or in some other region functionally connected with it) so that the tendency for BRVR normal brain treatments to raise the score still higher, particularly in positions 5 and 6 could be due to greater penetration of agent in those or associated regions. Such a model, however, seems to require some kind of <u>specific</u> mechanism - as though the exposure (including repeated exposure) to some antigen encourages

a particular pattern of differential neuroinvasiveness. The difficulty about specific mechanisms is that one would not expect them to work in the isogeneic combination i.e. BRVR-passed ME7 should not show a reduced neuroinvasiveness in BRVR mice, which it does. Such an autoimmune type of reaction would, however, be explicable in terms of responses to either sequestered antigens that have been exposed by scrapie, or to scrapie-directed antigens, or to normal self-antigens which have become immunogenic on account of an adjuvant effect of the agent (rather in the manner proposed for the immunogenic RNA in some hypotheses concerning the role of macrophage-processing of antigens in the immunological response (Pribnow & Silverman, 1967; Bishop, et. al., 1967; Fishman, 1970). The type of model one may envisage for scrapie pathogenesis in the periphery involving specific lymphoreticular reactions is as follows. Suppose that the agent is physically associated, in both the spleen and the brain, with a particular membrane structure (organelle or receptor) and that the injection of infected tissue causes the initiation of clones specific for that membrane structure (due perhaps to an adjuvant effect of the agent) then sooner or later the specifically sensitised cells will make immune responses of either a humoural or cellular type to these antigens wherever they are found. Such specific antibodies or cells would provide an agent with a highly convenient mode of transport to its target sites. In this way agent associated with a particular antigen in the donor could be carried to the same or a cross-reacting antigen in the recipient. (Perhaps the failure of some agents to cross species barriers e.g. TME to mice, could be due to the absence of a suitable cross-reacting antigen, Hadlow & Karstad, 1968;

Hanson, et. al., 1971).

A powerful argument against any such model is, of course, the frequently demonstrated failures of immunological manipulations to have any effect on scrapie incubation periods. However, the results described in SECTION 4 with prednisone acetate indicate that such effects are possible. If the population of cells concerned is very resistant to most immunosuppressive regimes, requiring something that borders on the level that will produce wasting disease, then many treatments may have failed to reach this level, especially when long chronic courses of treatment were attempted. On the other hand attempts to actively immunise in the case of an agent which used specific reactions to direct it to its target organ, may actually encourage shorter incubation periods, although these would not be detected if one was already using maximum doses in the interests of speedy results, and they certainly would not have any effect when the agent was actually delivered to the arget organ by an i.c. injection, as has sometimes been done in such experiments.

Another interesting possibility is that a condition such as that postulated above could sometimes progress to a genuine autoaggressive condition. Such may for instance be the case when incubation periods run into decades rather than months or years e.g. multiple sclerosis.

It has recently been shown that BRVR mice have the M_1 lymphocyte-surface antigen while BALB/c and C57BL mice have the M_2 antigen (Oliver, pers. comm.). Such antigenic differences could underlie the results obtained in these experiments, and investigation

of this possibility has begun.

Finally, it should be mentioned here that the notion of specific responses by the lymphoreticular system need not necessarily be confined to the well-known immunological responses: there is a growing feeling that these may only be rather specialised versions of a general morphostatic control system in the adult animal which governs matters like relative organ size and repair mechanisms. This will be dealt with further in the Final Discussion in SECTION 7.

SECTION 6

CHANGES IN THE DRINKING AND FEEDING HABITS OF MICE WITH SCRAPIE

CHANGES IN THE DRINKING AND FEEDING HABITS OF MICE WITH SCRAPIE

In the phase of experimental scrapie when the mice are ill there is a variety of obvious changes in behaviour. The details vary considerably depending upon the strain of host and scrapie agent. They can include: hyperexcitability, inco-ordination of gait, cessation of movement, somnolence, "freezing" postures when moved, circling behaviour and, more rarely, pruritis. However, the very variety and lateness of onset of these patterns consistent as they are within particular combinations suggests that they must be a consequence of secondary changes in brain physiology resulting from scrapie infection, and as such are not likely to repay close study for information regarding the primary lesion.

While the clinical phase of the disease occupies the final 5 to 10% of the incubation period, the rise in titre of the agent in the brain can be detected from about 1/3 of the way through incubation (Eklund, Kennedy & Hadlow, 1965; Dickinson, Meikle & Fraser, 1969; Dickinson & Fraser, 1969). Furthermore, the characteristic histological lesions start to appear in the brain about half-way through the incubation period with the host/agent combinations that have been reported (Fraser & Dickinson, 1968), though in some other combinations, preliminary evidence indicates a relatively later appearance of lesions (Fraser, pers. comm.). These general findings therefore raise the possibility of there being more subtle behavioural changes occurring much earlier in the development of the disease which could be detected by suitably sensitive means and may reflect more primary levels of agent damage. Reports of such early

Table 6.1 Some characteristics of the agents used in SECTION 6

Scrapie agent	Original source	Strain of mouse host	Genotype of host[e] (sinc)	Incubation period (i.c.)	Intensity of grey-matter vacuolation[f]			Intensity of white matter oedema[g]
					cerebellar cortex (tonsil)	hypothalamus (periventricular area)	septum	
ME7	Suffolk[a] sheep (natural case)	C57BL	s7s7	170 ± 2	+	++	+++	+
		(C57 x VM)F1	s7p7	240 ± 2	---	---	---	---
		VM	p7p7	332 ± 3	+	+++	++++	+/-
22A	Cheviot[b] sheep (experimental) SSBP/1 brain pool	C57BL	s7s7	460 ± 3	++	+	+	+/-
		(C57 x VM)F1	s7p7	571 ± 7	---	---	---	---
		VM	p7p7	195 ± 2	++	+	+++	+/-
79A[d]	Goat[c] drowsy (experimental)	C57BL	s7s7	150 ± 2	++	+	++	+++
		(C57 x VM)F1	s7p7	255 ± 2	---	---	---	---
		VM	p7p7	295 ± 1	+	+	++	++

a Zlotnik and Rennie, 1963
b Dickinson and Meikle, 1969
c Pattison and Millson, 1961
d Dickinson, 1970
e Dickinson, Meikle and Fraser, 1968
f Fraser and Dickinson, 1968; Fraser, 1971
g Fraser, pers. comm.

--- not given
+/- very low incidence and intensity

changes have already been made by Savage and Field (1965) and Heitzman and Corp (1968) using open field and emergence tests of behaviour. These psychological tests are, however, rather too cumbersome for routine use on a large scale and the physiological basis of them is poorly understood. What is required is an early and centrally controlled change in behaviour which is easily and reliably assessable, and which is well understood in neurophysiology. These criteria, are more satisfactorily met by drinking and feeding habits than by any other behaviour pattern and this SECTION described some changes in these parameters in mice with scrapie. There is a possibility that such behavioural changes could provide a basis for identifying the primary biochemical lesion involved in agent replication.

In this SECTION it will be shown that alterations in drinking behaviour occur with diverse strains of scrapie, genotypes of mouse, routes of injection and with different stimulating solutes. The changes are sufficiently consistent in these very different scrapie situations to permit generalisations to be made. Some instances of a less thoroughly explored change in feeding behaviour will also be described although it has less generality than that of the drinking change.

SPECIAL MATERIALS AND METHODS FOR THE DRINKING AND FEEDING EXPERIMENTS

In the combined accounts of numerous experiments which follow, the following inbred strains of mice were used: C57, A2G, VL, BALB, VM and (C57xVM)F_1. Animals were between 4 and 12 weeks old at the time of injection.

Strains of scrapie agent. These are shown in Table 6.1 which summarises some of their standard properties in C57, VM and F_1 mice, with some of the principle differences in their lesion type and distribution being illustrated.

Controls. The behavioural changes of the scrapie-infected animals were compared with those of controls which were contemporaries of the same age, sex and genotype. Two kinds of controls were used: uninjected animals, or ones that had been injected by the same route with normal brain supernates prepared from animals of the same genotype as the scrapie-donor. In no experiment was there any evidence that these injections of normal brain had any long-term effect on behaviour, and they certainly did not result in scrapie.

Measurements of changes in drinking and feeding. The mice were all housed in the environment which is described in SECTION 2 - no special provisions being made for this type of experiment apart from the use of more rigid cage racks to avoid the loss of water by dripping from the bottle spouts due to vibration. The quantities of food and water consumed were assessed by weighing the food-hoppers and water-bottles at intervals and calculating the average consumption per mouse per day. The details of the weighing schedules varied a little from experiment to experiment. Water bottles containing tap-water were weighed 1, 2 or 3 times a week. In most experiments this tap-water was temporarily replaced at intervals with a solution which had previously been shown to produce a degree of polydipsia in the particular strain of mouse. The periods of polydipsic stimulation were either 48 or 72 hrs repeated every week or every two weeks.

These periods of polydipsia (and accompanying polyuria) were well tolerated by most strains of mice (in some cases the period of trial was every two weeks for more than 600 days), but some, in particular the VL strain seemed to go into urinary collapse if stimulated too frequently. Such animals, with or without early scrapie, will drink as much as half their body weight per day.

Polydipsia-inducing solutions. Various solutions have been tried and the following were used in the experiments.

- 2½% sucrose in tap water.
- 1% sodium chloride in tap water.
- A mixture of 4% glucose and 1% saline.

The use of these generally doubled or trebled the fluid intake of specific strains of mice during the periods of exposure.

RESULTS

Ex. 6.1 The response of C57 mice to 2½% sucrose (72 hr. every 14 days) after a standard ME7 injection i.c. is shown in Fig. 6.1. The rapid, progressive decline in polydipsic response as compared with the continued high responses of the controls, began about 7 weeks after the injection and was therefore much earlier in onset than any of the clinical signs of illness. The period of illness is indicated by the horizontal bar on this and subsequent Figures. The normal daily consumption of tap water ad libitum by C57 mice in this laboratory is about 3.5g/mouse.

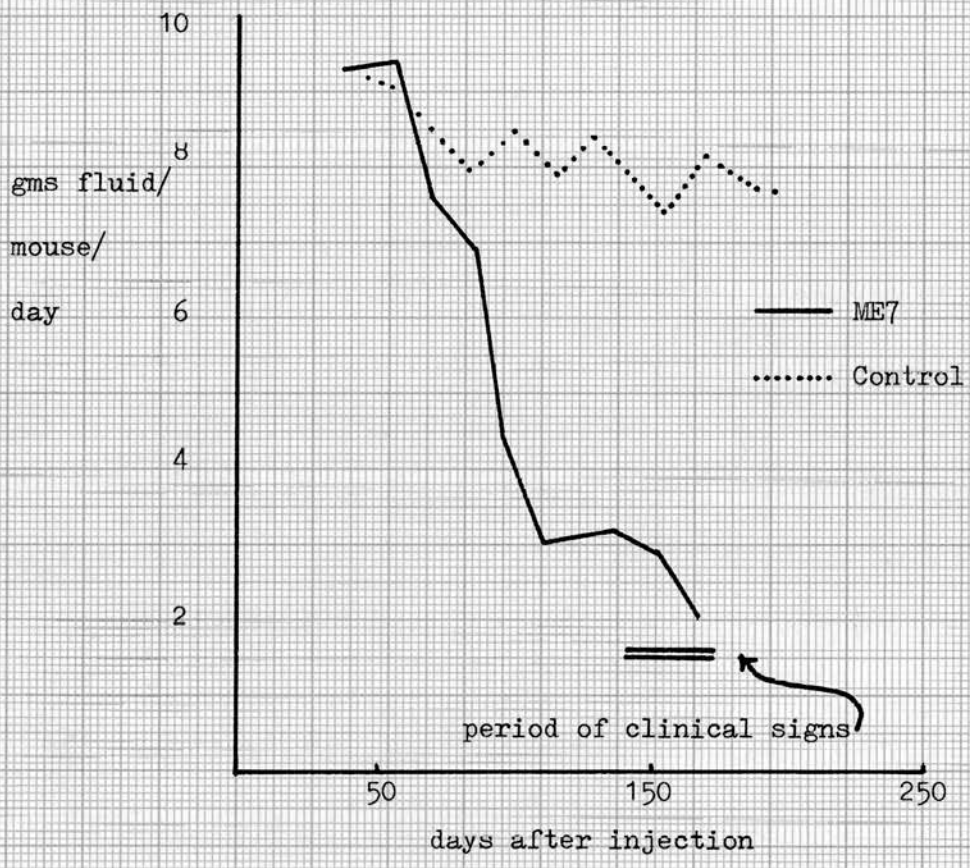


Figure 6.1 Drinking responses (2½% sucrose; 72 hrs. every 14 days) of 17 C57 mice after an i.c. dose of ME7 agent compared with the responses of 18 C57 controls injected i.c. with normal VM brain.

Ex. 6.2 The same agent(ME7) given i.c. to A2G mice produced an essentially similar change in drinking (Fig. 6.2).

Ex. 6.3 A standard 79A injection i.c. in VL mice, although a quite different agent/host combination, resulted in the same kind of change in drinking behaviour (Fig. 6.3).

Ex. 6.4 Both the agents so far described (ME7 and 79A) belong to the ME7-group, all of which give a shorter incubation period in s7s7 mice than in p7p7. Another experiment showed that the same pattern of drinking change could be obtained using the 22A agent in VM mice. This agent is very different from the ME7 agent in that it produces a shorter incubation period in p7p7 (i.e. VM strain) mice than in s7s7 mice, and its use therefore provided a check on the generality of the scrapie-produced behavioural change. Two different stimulating solutions were used in this VM experiment: 2½% sucrose or 1% NaCl in tap water (72 hrs every 14 days). The controls received the 1% saline only. Fig. 6.4 shows that the fall-off in polydipsic response was the same with both stimulating solutions and essentially similar to that described already in the s7s7 hosts with ME7-group agents.

Ex. 6.5 The experiments so far described in this SECTION used the i.c. route of injection. This necessarily resulted in some direct damage to the brain, so the next experiment used a standard ME7 injection by the i.p. route into C57 mice in order to avoid this potential problem. Fig. 6.5 shows the changes in response to 2½% sucrose solution and should be compared with the same agent/strain

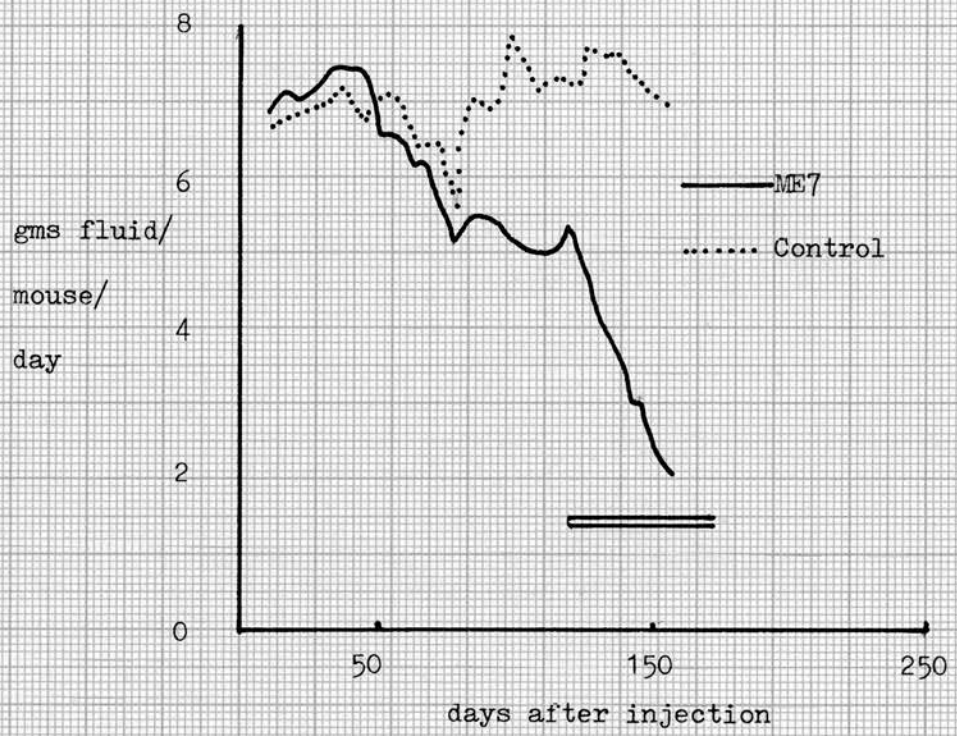


Figure 6.2 Drinking responses (4% glucose + 1% saline; 48 hrs. every 7 days) of 17 A2G mice, after an i.c. dose of ME7 agent compared with the responses of 24 A2G uninjected controls.

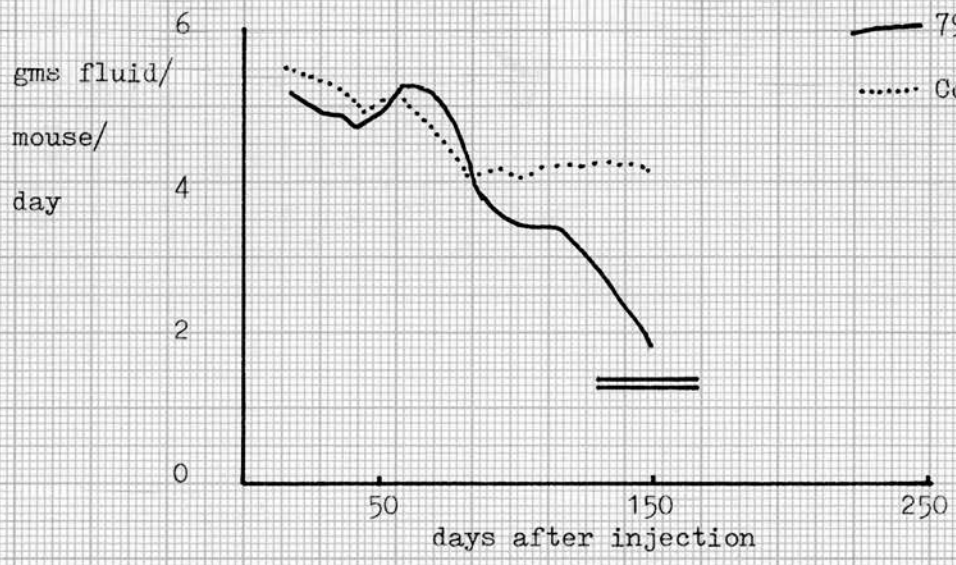


Figure 6.3 Drinking responses ($2\frac{1}{2}$% sucrose; 48 hrs. every 7 days) of 6 VL mice, after an i.c. dose of 79A agent compared with the responses of 6 VL controls injected with normal brain.

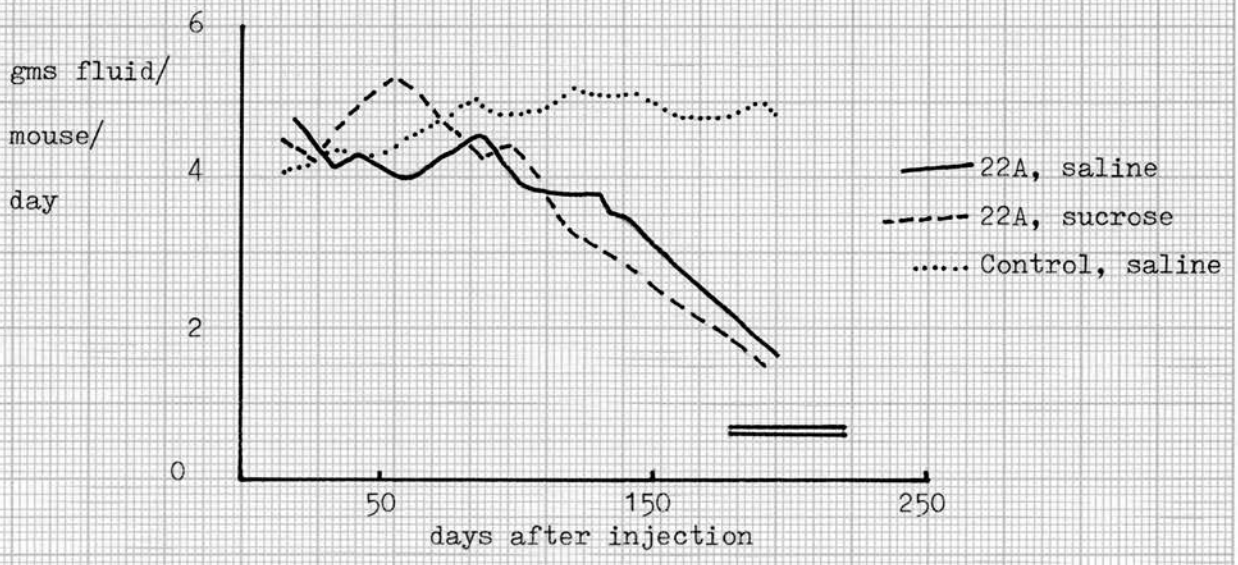


Figure 6.4 Drinking responses of three groups of VM mice (72 hrs. every 14 days). Responses of 11 VM mice to 1% saline after i.c. injection of 22A agent; responses of 12 VM mice to $2\frac{1}{2}$% sucrose after i.c. injection of 22A agent; responses of 5 uninjected VM mice to 1% saline.

Figure 6.5 Drinking responses (2½% sucrose; 48 hrs. every 14 days) of 18 C57 mice, after an i.p. dose of ME7 agent, compared with the responses of 18 C57 uninjected controls.

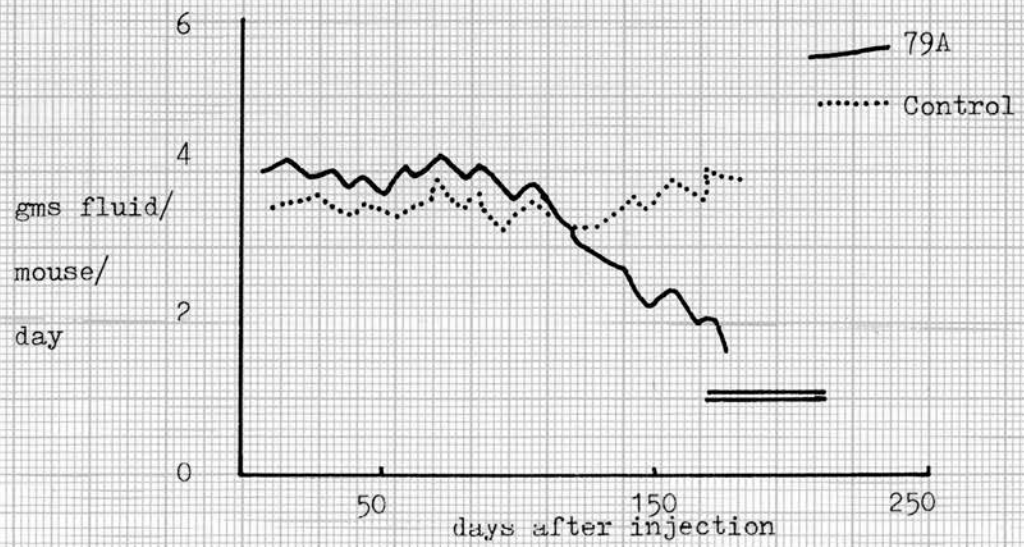


Figure 6.6 Drinking responses (tap water; average weekly consumption) of 12 VL mice, after an i.p. dose of 79A agent, compared with 12 VL uninjected controls.

combination using the i.c. route shown in Fig. 6.1. The delay in the drinking change and the prolonging of the incubation period were essentially of the same order: 60 and 55 days respectively.

Ex. 6.6 Another experiment using a standard 79A injection i.p. in VL mice, in which changes in drinking ordinary tap water alone were measured, gave similar results (Fig. 6.6). This, taken with the similarities in responses to sucrose and saline shown in Fig. 6.4, shows that the basic change is one in drinking and not a response to the solute.

Ex. 6.7 Figs. 6.7-9 show that there is an important association between the timing of the change in drinking and the action of the *sinc* gene in controlling incubation period. Figs. 6.7 and 6.8 show the effect of standard ME7 injections i.c. and the 22A agents respectively on the drinking habits of 3 genotypes of mouse, C57, VM, and their F_1. Fig. 6.9 shows the responses of the C57 and VM controls for these experiments - drawn separately for clarity. The controls show an essentially level polydipsic response over many months. All animals were stimulated with 2½% sucrose solution for 72 hrs every 14 days. A comparison of these graphs shows that the overall drinking changes correlate directly with the control that is exerted by the *sinc* gene upon the incubation periods in parental genotypes also being shown by the drinking responses, and in the difference in allelic interaction with the two agents, including the absence of dominance with ME7 and overdominance of s7 with 22A (SECTION 2.3a).

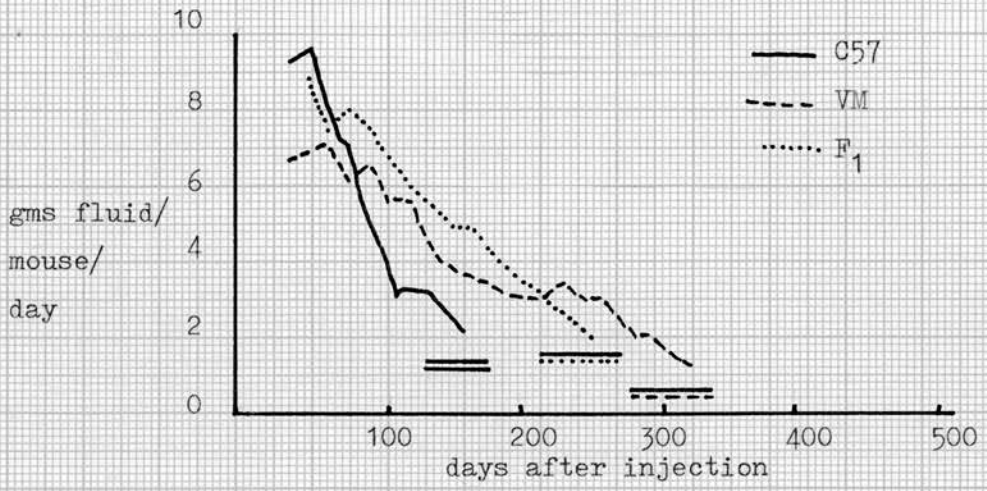


Figure 6.7 Drinking responses (2½% sucrose; 72 hrs. every 14 days) of three genotypes of mice after an i.c. injection of ME7 agent. Responses of 17 C57 mice; 12 VM mice; 18 (C57xVM)F_1 mice. Controls Fig.6.9

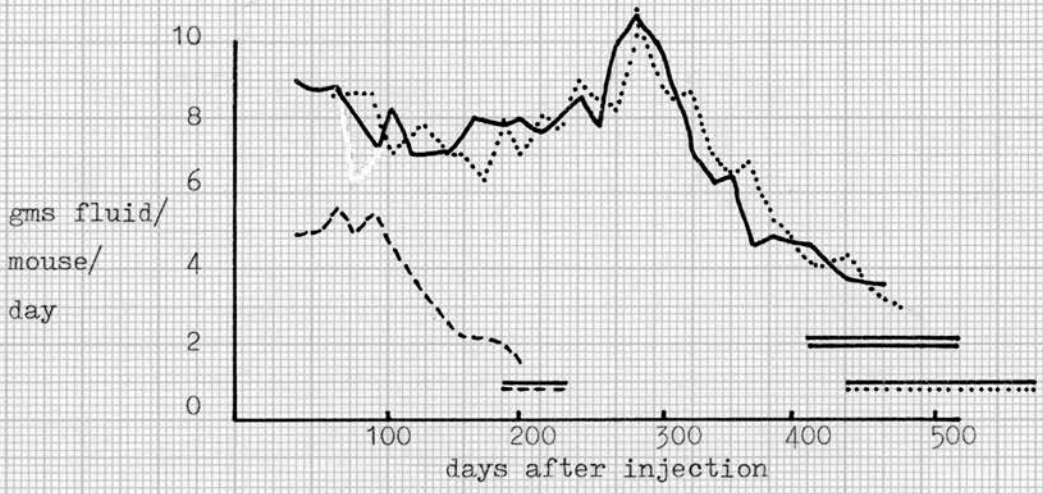


Figure 6.8 Drinking responses (as in Fig. 6.7) of the same three genotypes of mice shown in Fig. 6.7 after an i.c. injection of 22A agent. Responses of 12 C57 mice; 18 VM mice; 17 F_1 mice. Controls Fig. 6.9

Figure 6.9 Drinking responses (as in Fig. 6.7) of 18 C57 and 18 VM control mice, after an i.c. injection of normal VM brain.

Ex. 6.8 The central control of drinking and feeding behaviour is inter-related in mammals. Figs. 6.10-12 show some results from experiments in which the changes in weight of food eaten by scrapie infected mice were measured. Three different agent/host strain combinations are illustrated and they were chosen for investigation since remarkable changes in body weight had already been observed to occur in them. Injections of ME7 agent into A2G and BALB mice results in increases in body weight of mice of both sexes, while 22A agent in VM mice results in a long progressive emaciation. Measurements were made of changes in drinking, feeding and body weight in all three combinations.

The results in Figs. 6.10-12 are shown as a % of the control values as this permits a direct comparison of the timing of the changes in the three features measured. In all three combinations it can be seen that the changes in body weight are reflected by changes in feeding behaviour. Animals in the phase of great over-weight show considerable quantities of subcutaneous and peritoneal fat which is probably mainly responsible for the extra weight. Of note too in these ME7 systems, is the way in which the rise in feeding behaviour occurs at nearly the same time as the fall in drinking with a slight suggestion that the earliest phase of increased feeding is accompanied by temporarily increased prandial drinking. It should be strongly emphasised that these marked early changes in feeding behaviour, although very consistent for the agent/host strain combinations described, are not a general occurrence. While most mice show a sharp fall in weight during the late clinical phase, neither the sharp rise in weight nor the long progressive decline shown in these Figures

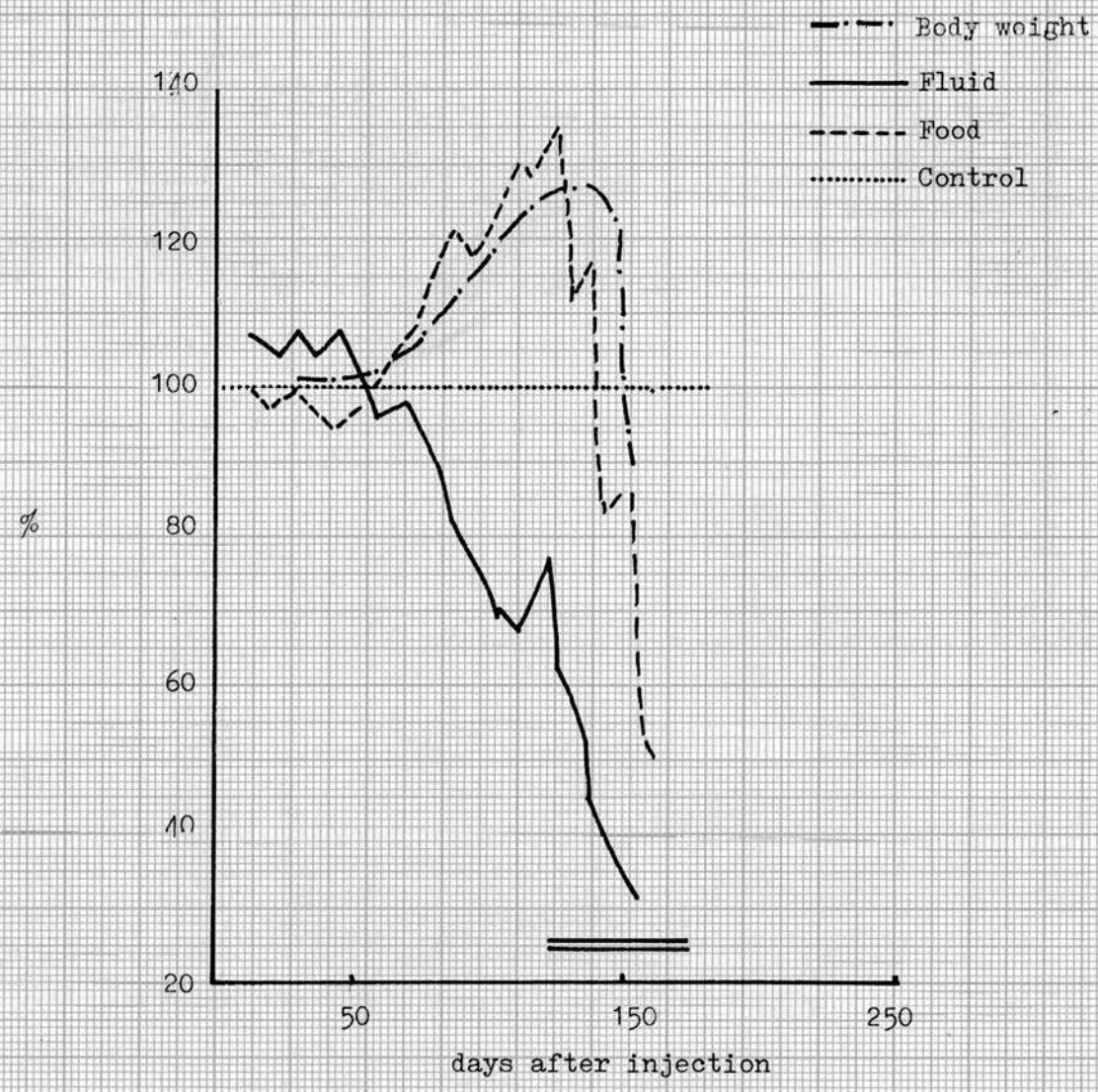


Figure 6.10 Drinking responses (4% glucose + 1% saline; 48 hrs. every 14 days) and the feeding of 17 A2G mice after an i.c. injection of ME7 agent, expressed as a percentage of the drinking and feeding of 24 uninjected control A2G mice.

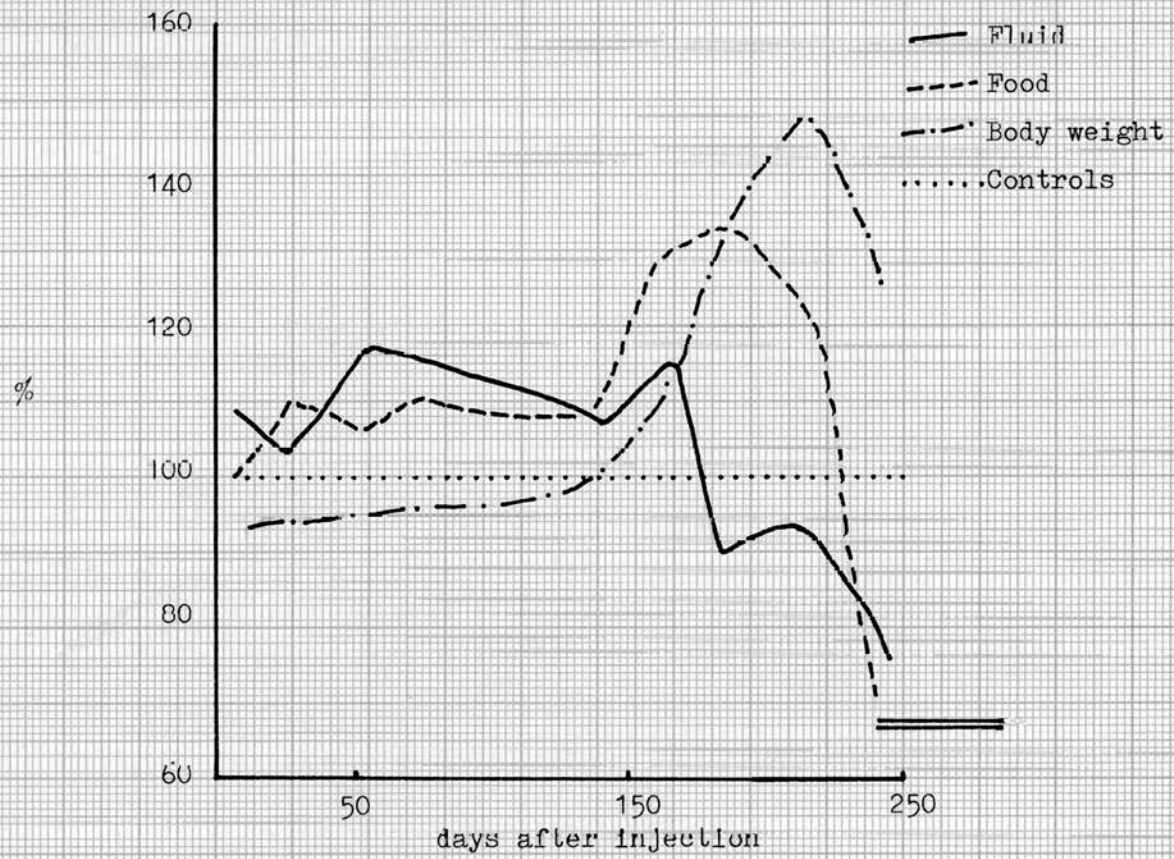


Figure 6.11 Drinking responses (2½% sucrose; 72 hrs. every 14 days), feeding and body weight of 23 BALB mice injected i.p. with ME7 agent, expressed as a percentage of the values shown by 24 control BALB mice injected i.p. with normal brain.

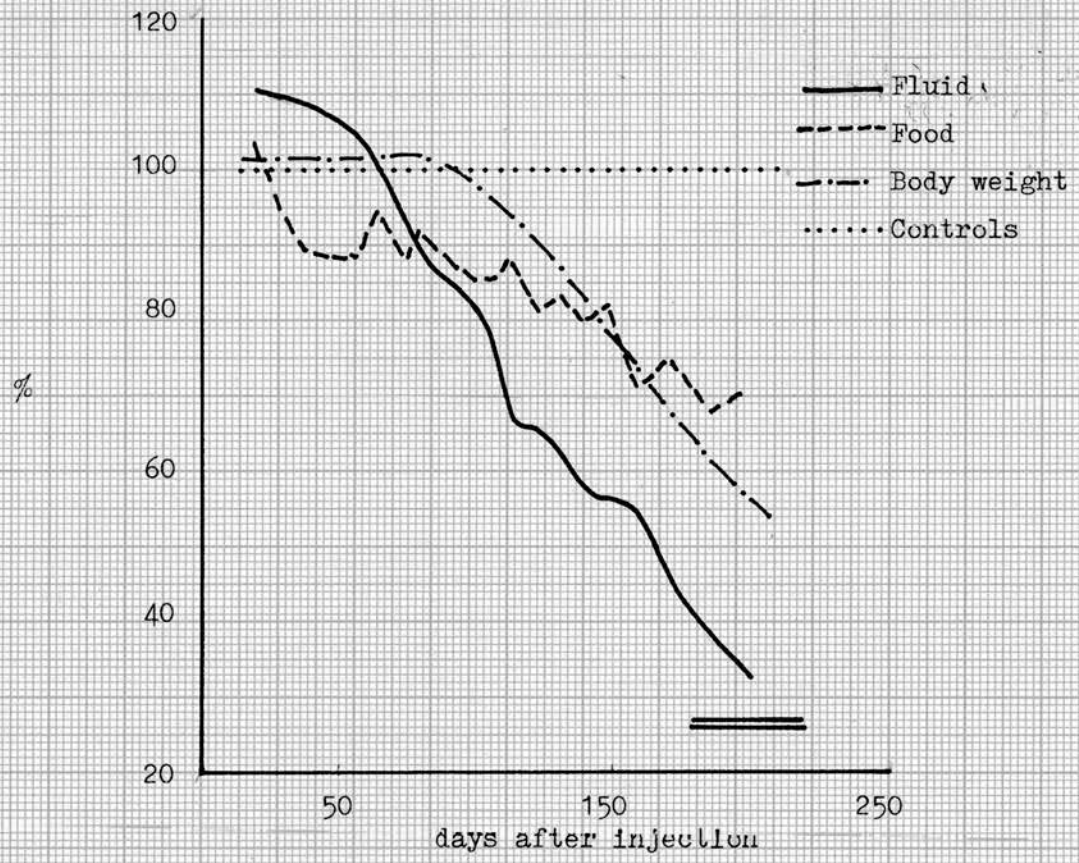


Figure 6.12 Drinking responses (2½% sucrose or 1% saline; 72 hrs. every 14 days), the feeding and body weights of the 23 VM mice shown in Fig. 6.4, injected with 22A agent i.c., expressed as a percentage of the values of the 5 uninjected VM mice on periodic 1% saline.

is common in the many combinations of agent and host that are used in this laboratory. This is not a case of the properties of a particular agent, since the increases in weight shown by the A2G and BALB mice with ME7 is not shown in numerous other strains of mice with this same agent. Moreover, C57 and F_1 mice which show no particular change with ME7 do increase in weight with 22A. Nor is it a property of the mice, since the VM mice which show an early decline in weight with 22A (Fig. 6.12) show a great increase in weight with the 22C agent (not shown). (See also APPENDIX 1).

The fact that A2G and BALB mice are increasing their food intake at the same time as they are reducing their fluid intake, further shows that the decline in drinking cannot be simply due to a progressive difficulty in holding the head up or any other such postural defect: both the food and water are supplied overhead and require a similar physical attitude to obtain them.

SECTION 6 DISCUSSION SCRAPIE MODIFICATION OF CENTRAL CONTROL OF DRINKING AND FEEDING

For a behavioural change to be of fundamental interest in the pathogenesis of scrapie certain criteria have to be met. Firstly, it must be the direct consequence of a primary lesion: early changes are more likely to fulfil this criterion than late ones. Secondly, it is of importance to be able to establish whether scrapie-associated behavioural changes are of central or peripheral origin. As already discussed in SECTIONS 3 & 4 there is considerable replication of agent in the lymphoreticular system and this precedes replication in the brain irrespective of the route of inoculation (Dickinson & Fraser, 1969). Apart from the finding of

interference in the lymphoreticular system with an arbovirus (Albrecht, 1970) no changes affecting the scrapie host's functions outside the central nervous system have yet been reported. The possibility remains, however, that there are a variety of peripheral functional lesions resulting from the agent's replication. Thirdly, the testing procedure that is used to detect changes in behaviour must not itself radically modify pathogenesis. Fourthly, since the study of behaviour is such a complex matter, the parameter chosen for study should preferably be already well characterised in fundamental physiological research. The change in drinking behaviour of scrapie mice, and to a lesser degree that of feeding, meet these criteria and so provide a basis for a potentially fruitful research programme.

To the question of whether or not the modified drinking response could reflect a primary lesion in the central nervous system, the earliness of onset and the wideness of the variety of host-agent combinations which show it, strongly support this possibility. That the change is not simply due to brain damage brought on by toxins present in the inoculum is shown by the different timing of the change when intracerebral (Fig. 6.1) and intraperitoneal (Fig. 6.5) routes of injection were used and also by the delay of nine months before intracerebrally injected 22A began to affect drinking in s7s7 and s7p7 mice (Fig. 6.8). The modified feeding response, considered apart from the drinking change, does not meet all the criteria needed to establish a good likelihood of being a direct consequence of a primary lesion. In the systems so far investigated (Figs. 6.10-12), the feeding changes are of a variety of kinds, and until more diverse

combinations have been studied, and the methods of detecting its onset have been refined, the matter cannot be resolved further. The data, are not, however, incompatible with primacy, since differences between the various agent/host combinations may be due to differences in the timing of the arrival of agent in the relevant brain centres. The earliness of the feeding changes and the fact of the inter-relatedness of the controls of drinking and feeding functions in the normal animal (Leibowitz, 1971) both justify the inclusion of the feeding change in the enquiry.

That the drinking change is due to central rather than peripheral lesions is made likely by the results for intraperitoneal injection and by the apparent close association with the basic effect of the sinc gene. If the effect of the agent on drinking had been peripheral, one would have expected the onset of the behavioural change to be at the same time irrespective of the route of injection, because it is known that the initial rise in titre of ME7 agent in the spleen of s7s7 mice is at almost the same time for both routes of injection (Dickinson & Fraser, 1969) and, by inference, the same would be expected in other peripheral sites, although no data are available. In fact, the delay in the onset of reduced drinking (Fig. 6.5, cf. Fig. 6.1) is almost the same as the increase in incubation resulting from the peripheral injection.

From the results where different sinc genotypes were used (Figs. 6.7-8) it is evident that variation in the timing of the start of the drinking change is to a very large extent controlled by the sinc gene. This gene appears to act on agent replication in each

tissue (Dickinson & Meikle, 1971). On the basis of the limited available evidence concerning the timing of replication in the brain of the various agents in the different genotypes used (Dickinson, Meikle & Fraser, 1969; and Dickinson, pers. comm), it seems likely that the drinking decline, the increased feeding (Fig. 6.10-12), the astrocyte hypertrophy and the vacuolation of brain tissues all commence at about the same time that agent replications start in the brain.

It is concluded therefore that the results indicate that the change in drinking (and possibly the changes in feeding) are due to some very early biochemical lesion in the central nervous system and not to some peripheral damage.

Seen in the light of present day neurophysiology these results have both theoretical and practical significance. At the theoretical level the identification of a biochemical lesion close to agent replication could be of the utmost importance in understanding the disease. While on the practical level, these results show that a large number of the techniques and theoretical models of the neurophysiologist are now available for scrapie research. Feeding and drinking are probably the best understood aspects of centrally controlled behaviour in mammals although there is still a great deal of disagreement about the details amongst research workers. The employment of some of the established techniques for modifying drinking and feeding in scrapie animals compared with their effects on normal animals might lead to the identification of the primary biochemical lesion.

It would not be profitable to speculate at great length upon all the theoretical and practical possibilities that are thus opened up: they are too numerous, but some tentative suggestions regarding the more important possibilities will be made. There are clear resemblances between the reported behaviour patterns in scrapie animals and the changes brought about by experimental stimulation or destruction of certain brain centres. The literature on this subject is now vast and has been reviewed recently by Morgane (1969) and Hoebel (1971). However, the generally benign nature of the observable lesions in scrapie, especially in the early stages of the disease are suggestive of some subtle kind of failure of cell communication such as synaptic derangement rather than extensive breakdown of function. It is also no longer proper to look for such lesions exclusively in the hypothalamus: the earlier views that feeding and drinking were controlled by activity and satiety centers in this region (for example, Anand & Brobeck, 1951; Anand, 1961; Hetherington & Ranson, 1940; Montemurro & Stevenson, 1957; Larsson, 1954) have been superseded by 'neural circuit' theories which involve limbic and other extrahypothalamic areas (Grossman, 1968; Grossman, 1969; Milgram, 1969; Lorens & Kondo, 1969; Singer & Montgomery, 1970). In any case, vacuolar lesions in scrapie mice (assuming that these also reflect the primary lesion, which they may not) show that damage occurs in many regions of the brain (Fraser & Dickinson, 1973) and hypothalamic lesions are relatively minor in some host-agent combinations (Dickinson, 1970; Fraser, 1971). One example of a recently developed set of techniques in neurophysiology which could be used in scrapie research is the

stereotactically controlled injection of putative transmitter substances and their respective anatagonists into discrete areas of the brain (e.g. Grossman, 1962; Margules, 1969; Leibowitz, 1970a, 1970b, 1971). Leibowitz has shown, for instance, that a decrease in drinking and an increase in feeding (cf. Fig. 6.10, 11) can be obtained in rats by hypothalamic injections of either an alpha-agonist (e.g. norepinephrine) or a beta-antagonist (e.g. propranolol HCl; see also Lehr, et. al., 1967). This work clearly provides some testable hypotheses for scrapie as an agent of synaptic derangement.

The behavioural changes reported here and the variety of signs which develop in the clinical phase are compatible with what one would expect to happen if the central nervous system was infected by an agent which slowly and progressively put out of operation a single class of synapse. There is an increasing number of putative transmitter substances and their respective post-synaptic receptors (acetylcholine, epinephrine, nor-epinephrine, 5-hydroxytryptamine, histamine, dopamine, gamma-aminobutyric acid, glycine etc.) some with a fairly localised distribution and function (e.g. dopamine in the neostriatum and tuberculum olfactorum) and others more widespread in the brain and cord (e.g. norepinephrine and 5-hydroxytryptamine, Fuxe, 1965). One may include also: prostaglandins, polyamines, phosphatidyl inositol, and some oligopeptides which have been suspected of various supporting roles in synaptic transmission (Sano, 1970; Smythies, 1972; Hadquist, 1970). A creeping derangement of such a system could probably be compensated for to some extent by brain repair and homeostatic mechanisms (Green, et. al., 1972), with the result that animals would show no obvious signs of upset until

the resources for compensation ran out, with a consequent outburst of terminal clinical signs. This type of hypothesis would allow for the numerous minor variations in behavioural signs due to agent/host strain combinations by postulating differential damage (as reflected in lesion profiles) as a result of different distributions of classes of synapses according to host strain and different rates and sites of penetration by each agent. A major difference in scrapie pathogenesis, however, such as that produced by different alleles of sinc could only be accounted for in these terms by a fundamental difference at the level of transmitter substance or synaptic mechanism.

The complete reversal of the incubation periods for the 22A-group and the ME7-group of agents in s7s7 and p7p7 genotypes (Dickinson, et. al., 1968; Dickinson & Meikle, 1969; Dickinson & Meikle, 1971) stems directly from the most primary effect so far known in scrapie, namely the interaction of the agent with a product of the sinc gene to permit agent replication. Further evidence that agent replication is restricted to a limited number of cellular sites, which may be specified by the sinc gene comes from evidence for competition between different scrapie agents during pathogenesis of mixed infections (Dickinson, Fraser, Meikle & Outram, 1972). In neural tissue, some of these postulated replication sites may be linked to ones affecting eating and drinking by only a short chain of cellular events. An examination of the s7s7, s7p7 and p7p7 mice in terms of synergisms and competition between scrapie agents and pharmacological treatments, for instance, could possibly expose this chain and thereby throw light upon the nature of the scrapie agent or its mode of replication.

A histological examination of the distribution of vacuolar lesions in A2G mice after i.c. ME7 scrapie at the time when the drinking and feeding changes are beginning is reported in full in Appendix 4. Briefly, it is shown that no lateral or ventromedial hypothalamic vacuolar lesions occur at this early period in this combination, and that the earliest vacuolations are found in the dorsal hypothalamus, the perifornicular region, the hippocampus and later in the amygdala and septum (paraterminal body). Had there been any marked association of lesion distribution with the classical lateral and ventromedial hypothalamic feeding and drinking control centres this would have constituted strong evidence for a direct relation between these lesions and the behavioural defects due to scrapie. The observed distribution, however, tells us very little and perhaps adds to the suspicion that these, the best known and most easily demonstrated CNS lesions of scrapie, have little to do with the primary damage and are themselves a secondary effect of something more fundamental. There are well defined instances in which sheep, given experimental scrapie and showing all the classical clinical signs, do not show any vacuolar lesions (Dickinson, pers. comm). This suspicion does not, of course, diminish their diagnostic and discriminant value in the form of lesion profile analysis, although it would reduce their relevance for a fundamental understanding of scrapie.

However, the observed distribution of vacuoles could still relate to the cause of the changed drinking and feeding behaviour, because regions like the hippocampus, amygdala and septum have all been implicated in theories of modulatory control of the hypothalamic

centres, although much less is known of their mode of action. The septum, in particular, has featured frequently in recent experiments demonstrating changes in drinking and feeding of rats after lesion (Singer & Montgomery, 1970; Harvey & Hunt, 1965; Sorensen & Harvey, 1971; Altman & Wishart, 1971). There are also sex-differences in the degree of response to septal lesions and this provides another possible reason for the effect of sex upon the incubation period of scrapie (Appendix 3). (Kondo & Lorens, 1971; Lorens & Kondo, 1971). Also of interest is the demonstration by Leibowitz that stereotactic administration of adrenergic drugs in the dorsomedial hypothalamus and the perifornicular region of the rat can affect feeding behaviour (Leibowitz, 1970). It can be seen that all the areas where early lesions occur in this particular combination of ME7 agent and A2G host strain have also been shown to have some potential function in the control of drinking and/or feeding, although apparently at one remove from the primary hypothalamic control centres. It remains to be seen if correlations will be found in other scrapie combinations which show different patterns of behavioural change and overall lesion profile.

The strong resemblances between the temporary patterns of behavioural change which Leibowitz and others have been able to produce in rats by stereotactic injections of adrenergic hormones their analogues and blockers, and the permanent and progressive changes shown by scrapie mice, suggests the possibility that the biochemical lesion produced by scrapie may involve the catecholamines. If these are also related to the action of sinc then they could be very interesting indeed.

There are other observations pointing to the possible involvement of these amines. The apparent effect (Ex. 4.15) of the catecholamine-depleting drug 6-hydroxydopamine is one. It is interesting that the motor defects reported in rats after 6OHDA injections along the medial forebrain bundle are similar to clinical signs observed in some scrapie combinations (e.g. i.c. 79A in DBA/2): weak, hypotonic hind limbs which are dragged behind, spinal flexion, hyperresponsiveness to stimuli, pronounced aphagia and adipsia (Smith, et. al., 1972). Ventricular injections of 6OHDA also cause reduced responsivity to polydipsic solutions of sucrose (Sorenson, et. al., 1972).

It has also been shown in the case of several virus encephalitides in mice that monoamine metabolism is seriously disturbed (Lycke, et. al., 1970). A great reduction in the levels of dopamine and norepinephrine have been reported in a case of Creutzfeldt-Jakob disease (Brun, et. al., 1971).

For the catecholamines to have any relation to sinc one would expect some use of them by cells of the lymphoreticular system since this gene appears to control the onset of scrapie replication in these tissues as well as in the brain. As yet there seems to be no strong evidence that epinephrine, nor-epinephrine (or dopamine) are implicated in 'immunological' functions, although another biogenic amine, 5-hydroxytryptamine, has (Devoino & Yeliseyeva, 1971). Also a mixture of epinephrine and propiomazine impaires immune responses (humoural and cellular) and causes runting when injected into neonatal rabbits (Henson, et. al., 1968; Henson & Brunson, 1969a) and

and prevents experimental allergic encephalomyelitis (EAE) in adult rabbits (Henson & Brunson, 1969b). Nor-epinephrine (an alpha-agonist) augments PHA-induced ^{3}H-thymidine incorporation by lymphocytes, while phentolamine (an alpha blocker) prevents this augmentation, so that it is conceivable that an alpha-blocking agent could alter cellular immune responses (Hadden, et. al., 1970). Lastly, by way of exemplifying the possible implication of these substances in immune responses, is the observation that the adenyl cyclase of mouse and rat lymphoidal cells is activated by both epinephrine and norepinephrine (Makman, 1971).

As a way of testing quite simply if scrapie impairs the metabolism or transmitter function of the catecholamines some experiments are in progress to investigate if the behavioural responses of scrapie mice to periodic peripheral injections of drugs known or suspected of acting via central mechanisms are in any way different from the responses of normal mice. Numerous alpha- and beta-agonists and antagonists were tested in a preliminary way on several strains of mice. At present two of them are being used. Amphetamine is a potent producer of transitory aphagia probably by beta-stimulation and inhibition of re-uptake of epinephrine. Its effect on the hyperphagia of A2G mice with ME7 is being tested. Thyroxine (apart from its many other effects) is also suspected of central action in the control of catecholamine function. Emlen, Segal & Mandell (1972), postulate that thyroxine may bring about 'receptor tuning' for nor-epinephrine, so that increased concentration of the thyroid hormone lead to an increased receptor sensitivity. Accordingly a change in responsiveness to the effects of thyroxine

on feeding and drinking behaviour is being sought in experiments with scrapie mice. Home Office permission has been requested in order to do scheduled drinking experiments in which scrapie animals will not be given food and water simultaneously, which should enable a distinction to be made between changes in prandial and physiological drinking.

This SECTION has reported a change in drinking behaviour in scrapie mice of wide generality which, especially when taken with the feeding change shown by a minority of combinations, suggests that an early biochemical lesion of scrapie interferes with central cellular communication using the catecholamines, epinephrine and nor-epinephrine. More direct evidence for this is being sought which could form the basis for an understanding of how scrapie agent could cause this kind of 'failure in communication'.

SECTION 7

FINAL DISCUSSION

Final Discussion

The results of SECTIONS 3-6 have already been considered in their respective Section Discussions, and so it only remains to ask if they can be synthesised into an overall model and if there are possible types of explanation other than the immunological one which has been generally adopted in this thesis.

The intention of the work reported has been to try to identify some new primary aspects of the pathology of experimental scrapie in the mouse and to explore them as far as possible in the time available. Tactical considerations - notably the need to take precautions against getting deflected into a time-consuming pursuit of one of the many secondary effects of the disease - dictated a policy of having several relatively independent lines of investigation in which any interesting findings would be tested for wide generality as soon as possible in a variety of agent/host strain combinations. Four such independent experimental lines have been described. In the case of those relating to the effects of age and a number of lymphoreticular-reactive drugs on the incubation periods of i.p. injected agents, the results can be easily harmonised in terms of imbalances of cellular components of the lymphoreticular system at the time of infection. An attempt has been made in SECTION 5 to extend this model to include the possibility that pathogenesis may also require some specific responses of an immunological kind. It was suggested that agent may be directed to its target sites by 'immunological aiming' derived from an initial sensitization to scrapie-associated donor antigens. However, it must be admitted that

while there is still doubt about the identity of the ME7 agent in the two sub-lines there must also remain some uncertainty that the modifications in pathogenesis were entirely due to donor-tissue components and not to two slightly different agents. In the end, of course, the question may hinge on just what constitutes an agent and what a host-component.

The behavioural changes reported in SECTION 6 have the strongest claim of all the results in this thesis to be closely related to the actions of the sinc gene. The evidence suggests that the drinking change is due to some progressive derangement of a function of sinc in the CNS, but the class of upset has not yet been identified. Reasons have been given for suspecting that they could be due to a breakdown in adrenergic synaptic communication, and it would clearly be of great interest if it could be shown that this was the sphere of operation of sinc. However, the association of scrapie with these amines is only very tentative and furthermore, their little understood involvement with the lymphoreticular system (See SECTION 6) precludes anything but the most speculative synthesis of the drinking observations with those of the age- and PA-effects.

The generality of the age-effect and the PA-effect has been tested in a variety of agent/host strain combinations but in neither case has this been done in a long-incubation period type of combination. It will be appreciated that to add a prolonging treatment (which most are) to a long-incubation period combination, using agent injected by a peripheral route, could lead to experiments in which the animals died of old-age before showing any clinical signs. In

such circumstances it would be necessary to resort to 'blind passage' and serial titrations at, say, six-monthly intervals, in order to detect the effects of treatment. These are amongst the next generation of experiments and therefore cannot be reported here. The results of this type of experiment would be expected to give some strong indications of whether or not the age- and drug-induced modifications of scrapie pathogenesis operated through some disturbance of sinc function. Meanwhile, however, it is justifiable to devise some experimental programmes on the assumption that sinc is implicated in these results and to look for indications of a common biochemical or physiological basis for all classes of phenomena reported in this thesis. Such an approach also requires a readiness to entertain several quite different kinds of explanatory models simultaneously.

Before considering some samples of these it is relevant at this point to refer to a recent finding of a kind which may have a bearing on some results in this thesis but which is too recent to assess. It will serve to illustrate the possibility that quite simple new discoveries can still put an entirely new complexion on previous findings. It has been shown that tissues, both from animals with scrapie and persons with multiple sclerosis, contain a replicating factor which causes a rapid decrease in the proportions of polymorphonuclear neutrophils amongst the circulating leucocytes of mouse recipients (Licursi, et. al., 1972; Carp, et. al., 1972). Collaborative investigations in this laboratory have shown this factor to be present in a wide range of scrapie agents.* Evidence indicates that this factor is not the same as the infective agent of scrapie and has properties differing from scrapie agents in important

* Pers. comm. Carp, Dickinson, Licursi and Taylor.

respects: whether it is an autonomous product of scrapie infection or only in specific secondary association with scrapie is not known. However, it must be assumed that this factor was present in most if not all of the experiments reported in this thesis. As its only known effect is to alter the differential leucocyte count it could clearly be involved in several of the responses, although whether it is relevant to the interpretation of any of the results cannot at present be judged. This finding shows how important it still is in scrapie research to emphasise operation descriptions of findings and to avoid a premature commitment to one particular explanatory model.

In fact, none of the results which have been described in previous SECTIONS point irrevocably to the immunological model which has so far been adopted. As an indication of other possibilities a brief description will be given of two alternatives which have some attractive features of their own.

The enzyme phenylethanolamine-N-methyl transferase (PNMT), which catalyses the transmethylation of norepinephrine (NE) to epinephrine (E), usually requires for its induction, very high concentrations of glucocorticosteroids. This is achieved in the adrenal (mammalian) medulla by a portal system which carries the steroid directly from the adjacent cortex. However, the conversion of NE to E also occurs in other organs (e.g. the heart and brain) without the benefit of these high concentrations. This is also the case in some E-secreting phaechromocytomas, and in other vertebrate classes in which the medullary and cortical components of the adrenals are anatomically distinct. There is some evidence that this enzyme can exist in

several forms with different induction thresholds. High levels of PNMT and hence of E-production can also be produced by injections of large doses of glucocorticosteroids. Such injections in neonatal rodents also result in the retention and even hypertrophy of the foetal extra-chromaffin tissue which surrounds the sympathetic chains of the trunk (e.g. the organ of Zukerkandl) and generally atrophies soon after birth. The function of this tissue appears to be unknown, but in view of the new-found associations between adrenal development and that of the thymus and hence the lymphoidal system as a whole (Pierpaoli and Sorkin, 1972b & c) it is possible that this tissue has a role in this sphere. This intriguing subject has been fully reviewed recently by Pohorecky & Wurtman (1971). These observations regarding the role of corticosteroids in the induction of a catecholamine-metabolising enzyme and in the hypertrophy of tissue containing it, make possible another type of model of the age- and PA-effects. Suppose that in both the neonatal mouse and in older animals after PA-treatment, there is an extra large volume of catecholamine-metabolising tissue in the abdominal cavity. Such tissue, by virtue of its resemblance to sympathetic neurons could act as a 'diversion' for i.p. injected scrapie agents - especially perhaps if the animals also have a greatly reduced lymphoidal system (which may normally be able to transport the agent away to other sites in the reticulo-endothelial system). Such a fate for the agent could be a form of 'inactivation' covered by the rubric in SECTION 3: 'sequestered in some alternative tissue where it cannot replicate, do damage or gain access to target cells'. This kind of diversion would at least result in longer incubation periods and perhaps lower

estimates of titre. Liberation of agent when such tissue eventually atrophies may result in the 'late cases' often found in experiments of this kind.

This model has certain attractions: it easily accommodates the PA-effect as a consequence of a temporary restoration of a neonatal condition; it provides an explanation for a loss in titre which does not depend on destruction of the agent; it is easily disproved by experimental investigation; PNMT is the kind of enzyme which the sinc gene could conceivably be involved with; and the connection with catecholamine-metabolism is obviously relevant in connection with the behavioural changes. However, it is difficult to see how such a model could provide for any degree of specificity such as seems to be required in the BALB/BRVR phenomenon. It is also perhaps improbable that such a large loss of activity with so many survivors could be obtained by such a mechanism.

Another hypothesis which is interesting in that it provides for specific reactions by elements of the lymphoreticular system but without invoking the conventional immunological responses is suggested by the discussions of Pierpaoli & Sorkin (1972a) in which they reopen the old question of the primary role of the lymphoidal system. Over the years there have been several objections to the prevailing view that the sole function of this system is that of detecting and removing exogenous foreign antigens (e.g. Yoffey, 1962; Loutit, 1962; Burwell, 1963; Burch, 1968; Burnet, 1970; Bullough, 1971). These writers insist in various ways that the well-known immunological functions of lymphocytes are simply specialised off-shoots of a more

primary function such as 'surveillance' or 'morphostatic control'. Such hypotheses provide attractive alternatives for the mode of scrapie involvement in lymphoreticular functions. It has frequently been pointed out by scrapie workers that the pathogenesis of scrapie has a certain inexhorableness, and some have remarked that it does not resemble a disease so much as a growth process (Dickinson, pers. comm.). Should it turn out, therefore, that functions like the maintenance of inherited bodily proportions and the readjustments of organs to stress or injury (e.g. the hypertrophy of one kidney after the removal of the other) is a function of the lymphoidal system, then it is not difficult to see that the linking of scrapie replication to this type of system could provide it with the regularity that is observed. This would be especially true if the system in question was of central importance in the maintenance of life so that it could not be tampered with without fatal results to the host. Particularly attractive from the theoretical point of view, is that the morphostatic hypotheses require the operation of various tissue-specific affector and effector molecules, for which more and more potential candidates have recently been found. The affector molecules are envisaged as informing the central control (?thymus) of the status quo (and hence of changes to it), while the effectors would direct any necessary adjustments, (e.g. by restarting cell-division in appropriate tissues). Amongst the best known substances of these types at the present time are the lymphokines such as lymphotoxin, macrophage migration inhibitory factor, macrophage aggression factor, transfer factor, skin reactive factor, the interferons, tissue-specific chalones, and the wide range of substances extractable from salivary glands of which the best known

is the nerve growth factor. An association of scrapie with one or more of these types of informational molecules, whether at a simple physical level, or more subtly by something like episomal interference of their transcription (in the manner of lysogenic phages), could provide a degree of tissue specificity which would permit 'immunological aiming' and the general repeatability of lesion profiles. In fact, if scrapie agents were molecules very similar to these kinds of information molecules it could account for their ability to evade the host's immunological responses. It would also permit the construction of interesting cybernetic models for agent replication. For instance, the overloading of a homeostatically controlled system with exogenous informational molecules could lead to positive feedback oscillation in which the overswing responses made during the attempts to restore the status quo led to the production of more agent by the host. This type of pathogenic pattern should be detectable by looking for oscillatory phenomena in the preclinical phase in a variety of host systems (including behaviour patterns and perhaps EEG cycles).

It is stated in the introduction to this thesis that there is as yet no agreed methodology or theoretical framework in the field of the 'slow-viral encephalopathies' and in such circumstances it is dangerously easy to indulge in speculations beyond the point of practical scientific usefulness. It is important therefore, to close this Final Discussion on a more sober and realistic note. A number of findings have been reported which show that it is possible to modify the pathogenesis of peripherally injected scrapie agents in mice, both in terms of incubation period and lesion profile. This contrasts with the much greater difficulty in doing so with centrally

injected agents, although the effects of actinomycin D on incubation periods and of donor tissues antigens on i.c. profiles (Outram, Fraser & Wilson, 1973) look promising. However, this relative ease of modification of peripheral pathogenesis raises the possibility that the methods are operating on steps that are not controlled by the sinc gene, so that although they may offer some hope for the discovery of prophylactic treatments by throwing light on the early stages of infection, they may be less useful for elucidating the nature of agent replication, and hence for the discovery of cures. Alternatively this relative ease of manipulating peripheral pathogenesis may simply be due to a greater accessibility of the extraneural sites of interaction between agent and sinc gene, in which case further investigations of these findings could result in an identification of the normal role of this gene and hence of the nature of its relationship with scrapie agents.

APPENDICES 1 - 5

APPENDIX 1. BODY WEIGHT CHANGES IN MICE INFECTED WITH SCRAPIE AGENTS.

From time to time there have been reports of body weight changes in scrapie-affected sheep (Stamp, 1962; Daniel, 1971) and mice (Field, 1969). With outbred populations, conspicuous examples of over- or underweight occur in isolated individuals only. With inbred populations of mice, however, they appear to characterise certain agent/host strain combinations. In the experience of these laboratories such weight changes are the exception rather than the rule - most scrapie infected mice showing only a brief terminal phase of rapid loss in weight. Figure A1.1 shows the body weight data for a number of agent/host strain combinations. They illustrate both the common pattern (e.g. 79A in A2G and BALB mice and ME7 in C57 mice) and the occurrence of large increases in weight well before the terminal phase (ME7 in A2G and BALB mice and 22C in VM mice) and long periods of a slow decline in weight (22A in VM mice).

The physiological basis of these weight changes is not known. In the case of A2G and BALB mice injected with ME7 agent, it has been shown that the increase in weight is accompanied by increased food consumption (SECTION 6) and it is reasonable to assume that one is the cause of the other. However, it is possible that there are also metabolic modifications such as changed metabolic rates, or raised setting of homeostatic controls of body weight, or increased conversion of carbohydrates to lipids. These alternatives have not yet been investigated.

Perhaps the most likely root cause of these agent/host

strain differences in body weight responses (as in the case of lesion profiles), is a particular pattern of agent infection in the brain. Positive evidence for this is not yet available however.

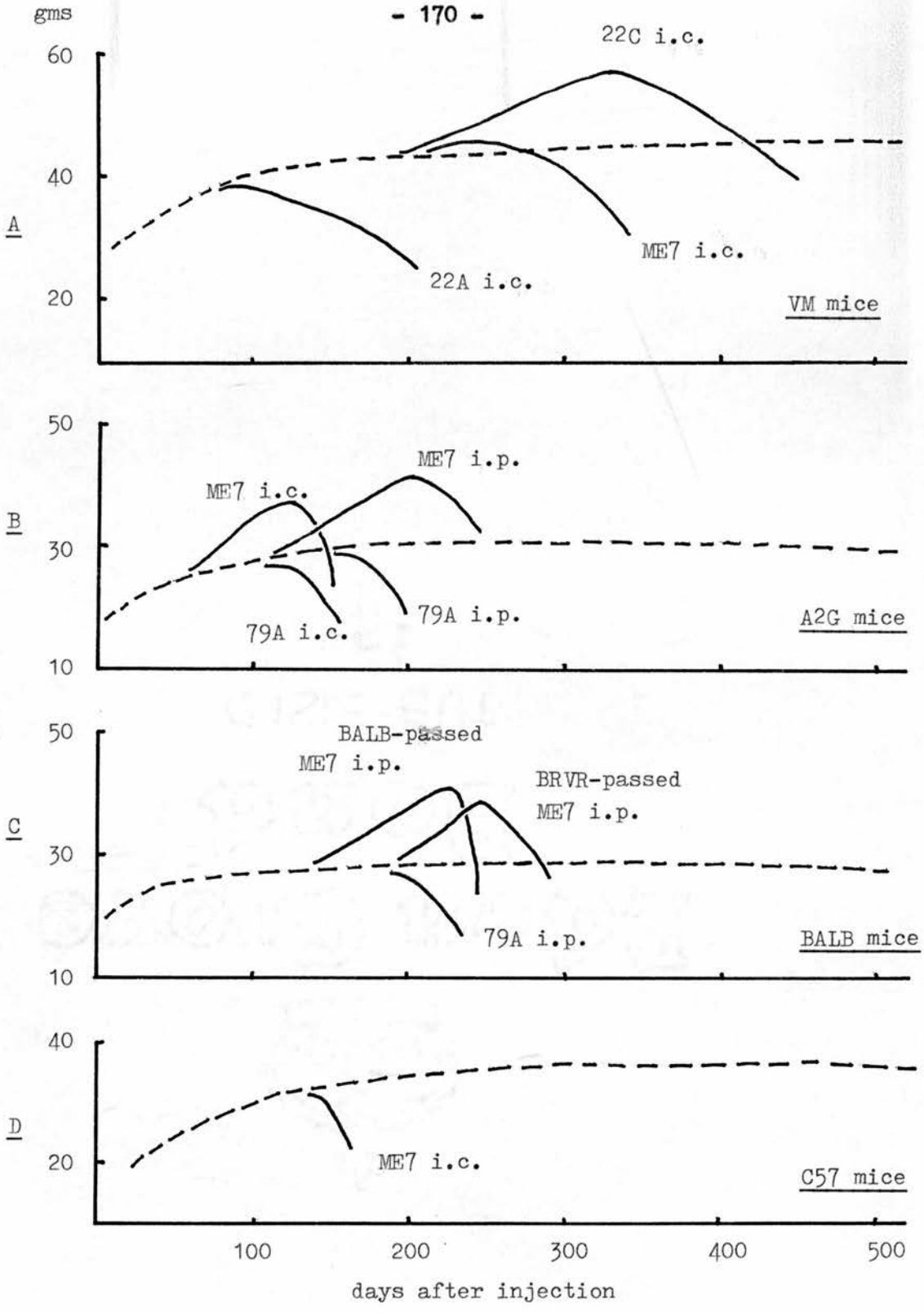


Figure A1.1 Body weights (gms) of four strains of mice injected at weaning with various strains of scrapie using the i.c. or the i.p. route. Body weights of controls shown by broken line.

APPENDIX 2. AN INVESTIGATION OF THE EFFECT OF A SINGLE S.C. DOSE OF 500 mg/kg OF PREDNISONE ACETATE ON THE WEIGHT OF THE THYMUS AND SPLEEN, AND THE DIFFERENTIAL LEUCOCYTE COUNT IN VL MICE

Large doses of prednisone acetate (PA) have been shown to modify the pathogenesis of scrapie agents in mice (SECTION 4). Cortisone acetate has been shown to have a profoundly depressing effect upon the weight of organs of the lymphoreticular system and upon the numbers of small lymphocytes in the blood (e.g. Elliott & Sinclair, 1968). Since such an effect could be the basis of the prolongation of scrapie incubation periods by PA, the following preliminary investigation was performed to find the gross effects of this steroid on the lymphoreticular tissues of VL mice.

Materials and Methods. Male and female VL mice ranging in age from 20 to 100 days were injected s.c. with PA (500 mg/kg suspended in isotonic saline) or saline only. Every individual was weighed at the start and at weekly intervals until killed. Groups of animals, each including a range of ages, were killed on the 4th, 7th, 14th and 21st day after the steroid injection and the following data obtained:

- body weight at the time of death;
- weight of spleen, thymus and brain;
- two blood slides (retro-orbital) for differential leucocyte count;
- peritoneal washout.

Blood slides were stained with Wright's stain and differential counts made of 200 leucocytes per slide using the following

categories: small lymphocytes, large lymphocytes, polymorphonucleocytes and monocytes.

The peritoneal cavity was filled immediately before killing with 2 ml. of heparinized saline. The abdomen was gently massaged, the mice killed by cervical fracture, the peritoneal contents pipetted into plastic containers and kept ice-cold until counted using a Neubauer chamber. A distinction was made between 'small cells' (= mostly small lymphocytes) and 'large cells' (= large lymphocytes, and polymorphonucleocytes).

The spleen, thymus and brain were removed, blotted and weighed.

Results. The PA injections had only a slight and transitory effect on whole body weight and none on brain weight - these results are not recorded here.

Fig. A2.1 shows the spleen weights before and after PA treatment. These were severely depressed in females for at least 7 days, and in males for between 4 and 7 days after injection. The thymus weights of the same animals are shown in Fig. A2.2. The normal decrease in weight with age is well-shown and the depressive effect of the steroid injection is apparent for at least one week and up to two weeks in the case of one female. The degree of depression appears to be maximal irrespective of age.

Differential leucocyte counts are shown in Fig. A2.3. The values for monocytes are not shown but remained within the .5-1.0% mark throughout. No significant modification of the proportions of large

lymphocytes were detected either. However, there was a marked decrease in the proportion of small lymphocytes and a corresponding increase in polymorphonucleocytes. Although no total count was performed there can be no doubt that the increased proportion of polymorphonucleocytes also represents an absolute increase in numbers: sometimes it was difficult to find any small lymphocytes on the slide at all while polymorphs were densely distributed all over the slide.

Peritoneal washouts after the s.c. PA injection showed the same initial depression in absolute number (Fig. A2.4) with evidence of considerable increase in numbers of the larger cells (probably polymorphonucleocytes) in some individuals by one week after injection. The small lymphocytes, however, remained depressed even three weeks later, suggesting that prolonged courses of steroid injection could keep the peritoneal cavity depleted of these cells for a long period.

These results confirm that PA has the same general lymphoreticular depressing effects that have been reported for cortisone acetate. The evidence of a long term unbalancing effect upon the proportions of peritoneal cell types supports the models for the PA-effect which are described in SECTION 4. Further investigation of the modification of scrapie incubation periods by PA, arachis oil, 6-hydroxydopamine and phytohaemagglutinin (SECTION 4) will obviously entail more detailed investigations of the kind described in this APPENDIX.

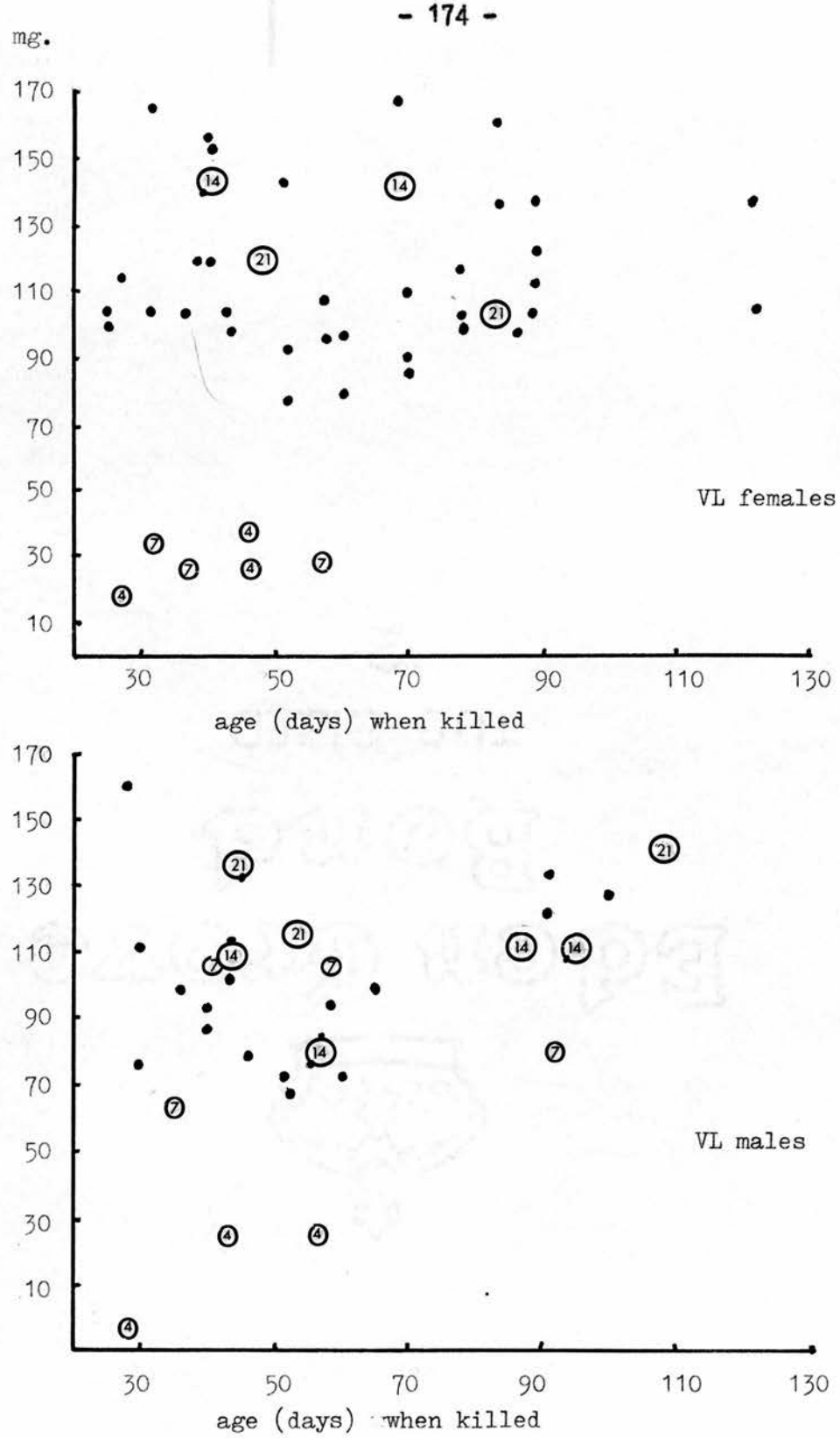


Figure A2.1 Spleen weights (mg.) of female and male VL mice at various ages from weaning to about 4 months old.

The ringed numbers represent the spleen weights of mice when killed 4, 7, 14, or 21 days after a single 500mg/kg dose of PA.

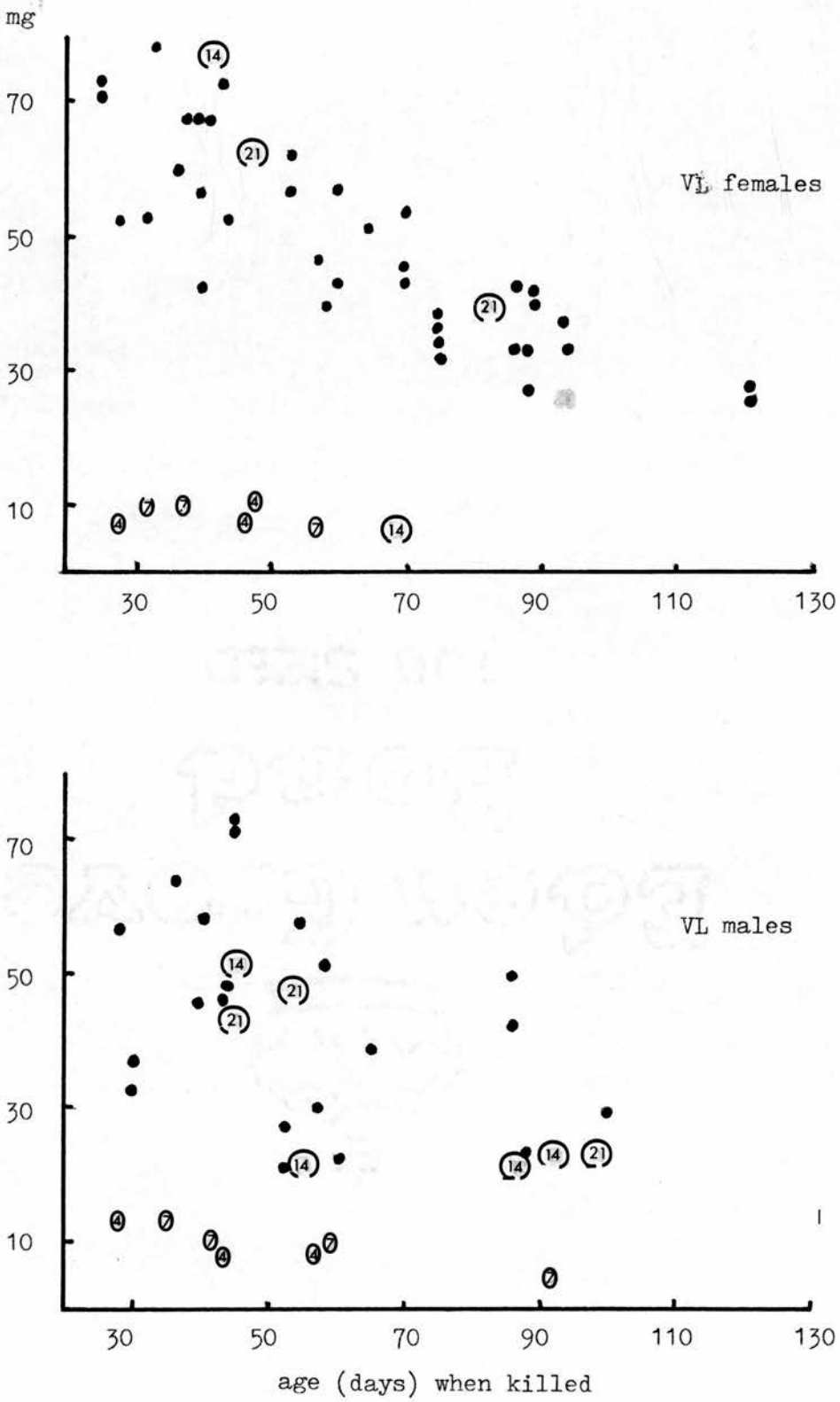


Figure A2.2 Thymus weights (mg) of female and male VL mice at various ages from weaning to about 4 months old.

The position of the ringed numbers represents the thymus weights of animals treated with 500 mg/kg of PA and the number indicates how many days after the steroid injection the individual was killed.

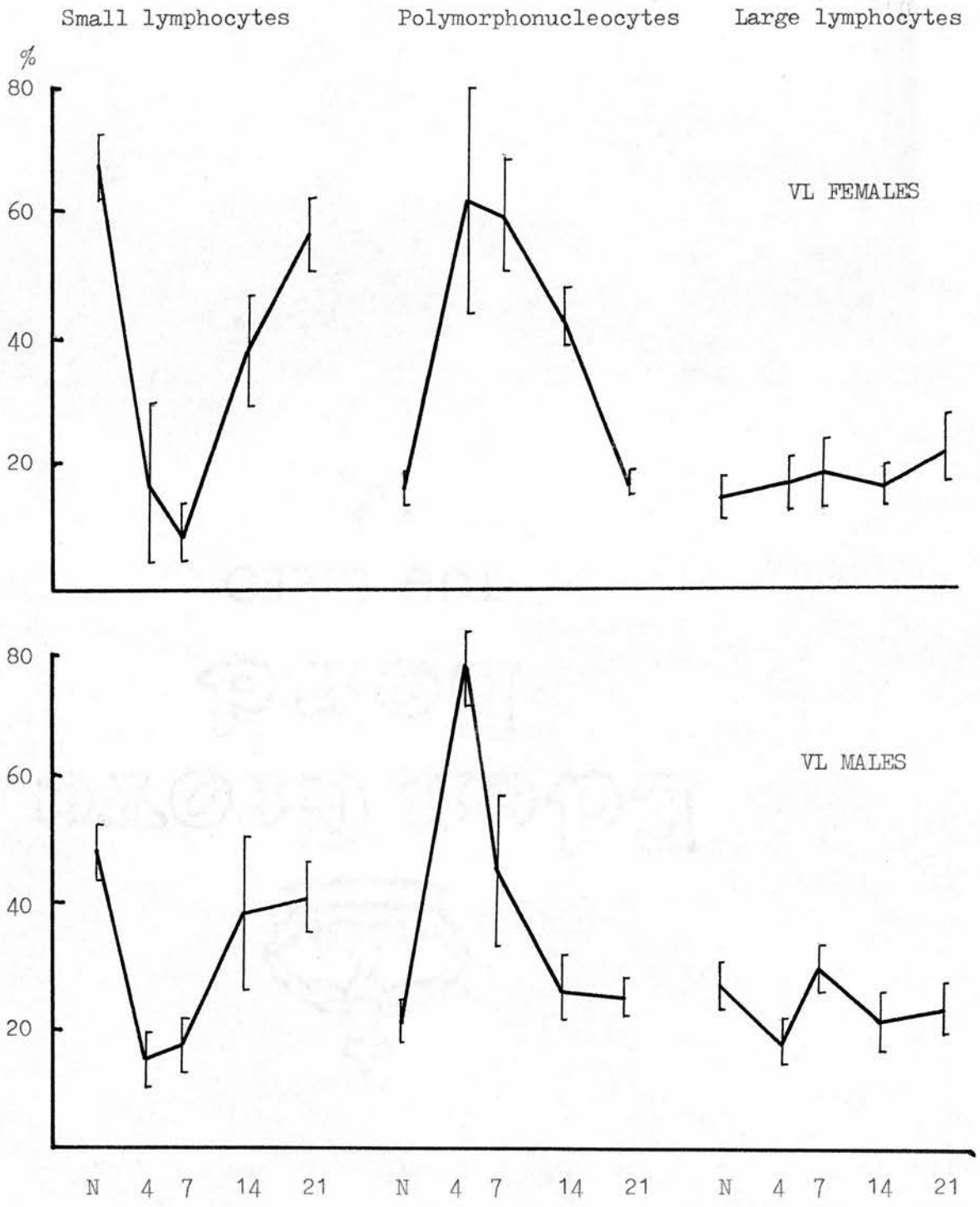


Figure A2.3 Differential leucocyte counts in the blood of VL mice normally (N) and 4,7,14 and 21 days after a s.c. dose of 500mg/kg PA.

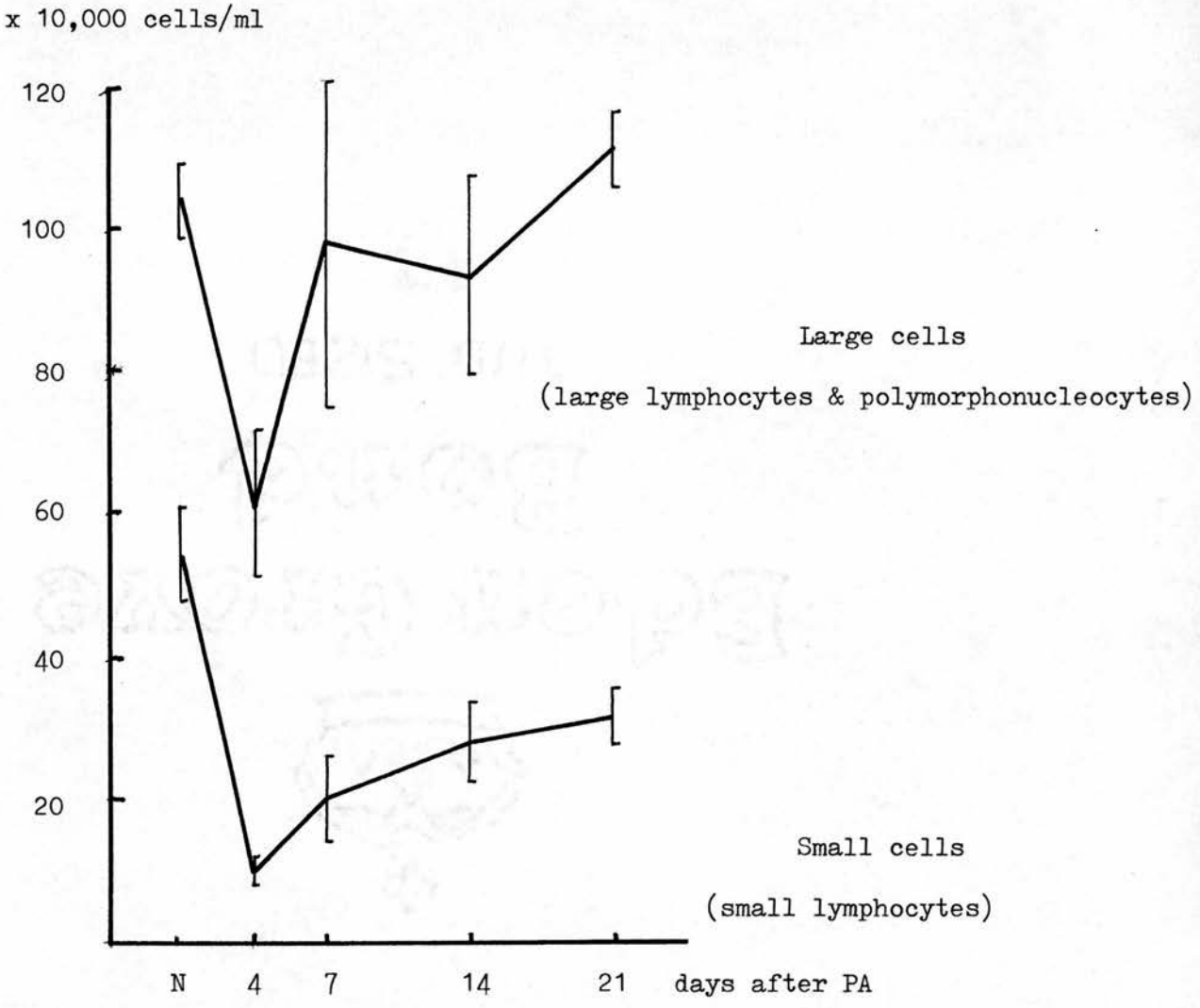


Figure A2.4 Cell counts in 2ml washouts of the peritoneal cavity of normal (N) and PA-treated VL mice, 4,7,14, and 21 days after injection.

APPENDIX 3 THE EFFECT OF SEX OF RECIPIENT ON THE INCUBATION PERIOD OF DIFFERENT AGENTS

Some early results using the 79A agent by the i.p. route showed a marked difference in incubation period according to sex. This prompted an analysis of the results from numerous experiments performed in previous years in these laboratories and using a variety of agent/host strain combinations. The incubation periods from several different experiments performed at different times, but using essentially similar operational conditions, were combined to obtain the overall means which are shown in Table A3.1. The results from other subsequent experiments especially using 79A have been added. The significances of the incubation period differences were obtained by simple t-test.

Comments It can be seen that incubation periods are usually significantly longer in males than in females for the agent/host strain combinations that were investigated. However, this was not the case for the ME7 agent in several genotypes and using both the routes of injection, although the mean incubation period for males tended to be longer than that of females at high dilutions in titration experiments.

The cause of this difference is not known at present. A careful examination of the laboratory records excludes certain possibilities such as: bias due to the strain or sex of donor, or to a scoring tendency of certain observers. The magnitude of the sex-effect depends upon the strain of agent and recipient, and the route of injection.

It is not known if the generally prolonged male incubation periods as compared with those of females represents different operational titres, different rates of replication, or different host resistance to damage leading to a delay in the onset of clinical signs. A comparison of the lesion profiles (not shown) for males and females in some of the combinations indicates that they are very similar and so does not suggest any preference for any of the above possibilities.

It is perhaps worth noting, however, especially in view of the emphasis that has been laid in this thesis upon the possible association of scrapie replication with immune functions, that female mammals generally show stronger immunological responses than males as well as higher incidences of autoimmune conditions and sequalae, and diseases suspected of immunological involvement such as Multiple Sclerosis (Poskanzer, 1965; Graff, et. al., 1969; Galton, 1967; Dresser, 1962). It could be therefore, that the more efficient immune system of females which stands them in good stead for conventional infections acts against them in the case of agents which make use of the immunological system for their pathogenesis.

However, since nothing is as yet known about the rates of replication of agent in the two sexes, or even of the comparative titre in terminal brains, other possibilities remain. For instance, it is known that there are pronounced differences according to sex in feeding and drinking responses after damage to brain areas, in particular the septum (Kondo & Lorens, 1971) so that it is possible that identical titres of agent in animals of either sex could cause a different timing of the onset of clinical signs and hence of estimated incubation period.

EFFECT OF SEX OF RECIPIENT ON THE INCUBATION PERIOD OF DIFFERENT AGENTS

Agent strain	Mouse strain	Route of injection	Incubation period		P
			females	males	
79A	RIII	i.c.	136±1	140±1	XX
	C3H		138±2	141±1	XX
	SM		148±1	150±1	-
	VL		151±1	155±1	XX
	C57		152±2	157±2	-
	A2G		160±1	166±1	XX
	BALB		161±1	166±2	XX
	RIII	i.p.	189±1	195±1	XXX
	C3H		195±1	202±2	XXX
	SM		204±1	207±2	-
	VL		205±1	221±1	XXX
	C57		201±2	207±3	-
	A2G		197±1	203±1	XXX
	BALB		227±3	232±3	-
ME7	C57	i.c. 10^{-2}	159±1	156±1	-
		10^{-3}	163±1	166±2	-
		10^{-4}	180±3	180±2	-
		10^{-5}	192±3	200±4	-
		10^{-6}	220±6	233±4	-
	A2G		161±2	166±3	-
	VM		322±3	326±2	-
	F_1 (s7p7)		244±2	246±2	-
	C57	i.p.	279±4	282±4	-
	A2G		232±3	235±3	-
22C	C57	i.c.	174±1	181±1	XXX
	VM		433±10	434±4	-
22A	C57	i.c.	437±4	464±5	XXX
	VM		198±3	206±2	X
	F_1 (s7p7)		545±9	579±9	XX
	F_2 & F_3(p7p7)		210±2	218±3	X
	F_2 & F_3 (s7s7 and s7p7)		511±8	546±6	XXX
	VM	i.p.	296±2	317±5	XXX

(- = P,>0.05; X = P, 0.05-0.01; XX = P, 0.01-0.001; XXX = P<0.001).

APPENDIX 4 HISTOLOGICAL EXAMINATION OF THE BRAINS OF ME7 INJECTED A2G MICE AT THE TIME OF ONSET OF CHANGES IN THEIR DRINKING AND FEEDING BEHAVIOUR (See SECTION 6)

The time at which drinking and feeding changes start to occur is about the same as the stage at which brain lesions are appearing, and so the pattern of onset of the vacuolar lesions has been examined in detail in one host/agent combination. On the assumption that this pattern could reflect the distribution of the fundamental lesion due to scrapie, it was thought possible that some correlation might be found between it and the changes in drinking and feeding behaviour which are characteristic of this particular agent/host strain combination (SECTION 6, Ex. 6.8). The most obvious expectation was that the lesions would occur in the lateral (LH) and ventromedial hypothalamus (VMH) which contain the centres for the direct control of drinking and feeding in mammals.

Material and Methods. A2G mice were given standard i.c. injections of ME7 agent and then killed in groups of three, 60, 70, 80, 90 and 100 days later. Standard cross-sections were prepared as usual (SECTION 2) and in addition serial sections of 2 of the 3 brains in each group. These haematoxylin and eosin stained preparations were examined for vacuolar lesions and their distribution marked on diagrams.

Results: There were no vacuolar lesions 60 days after injection, but from 70 days onwards they appeared and increased in severity. The total lesion scores (SECTION 2) were as follows:

60 days	0.0 ± 0.0
70 "	2.3 ± 0.7
80 "	6.7 ± 0.7
90 "	13.0 ± 3.0
100 "	19.0 ± 0.0

The normal total lesion score for this particular combination in terminal cases (175 days) is 26.2 ± 0.6.

The earliest vacuolar lesions were present in the 70 day group in the dorsal part of the hypothalamus, the hippocampus and the caudate nuclei. They were very small and widely dispersed. By 80 days it was apparent that the hypothalamic vacuolation was centred on the regions immediately dorsal to the ventricle, in the nucleus dorso-medialis, and more laterally in the periformicular regions and the zona incerta. Hippocampal, caudate and septal lesions appeared or became more dense. These distributions were found at 90 days also, but with more intensity. By day 100, slight vacuolation was also apparent in the amygdala and the central part of the medulla oblongata; no vacuoles were found in the cerebellum. At this time the hippocampal and hypothalamic lesions were very conspicuous but those of the septum and caudate were still small and scattered.

Discussion. The most significant observation in these data is that although the drinking and feeding changes are well-advanced by 100 days after i.c. injection in this particular agent/host strain combination there was no sign of either LH or VMH vacuolation. This

could mean either than these centres are not primarily involved in the behavioural change, or that the vacuolar lesions do not accurately represent the distribution of a more fundamental biological lesion that is involved. It remains possible that either or both these hypothalamic centres are malfunctioning but without yet having proceeded into vacuolation as they do later in the incubation period.

However, of the regions where early vacuolation does occur, three of them (hippocampus, amygdala and septum) have been implicated in 'neural circuit' theories of control i.e. they have a modulatory function with respect to the hypothalamic centres (Singer & Montgomery, 1970). Furthermore the perifornicular region in the rat is sensitive to both alpha- and beta-adrenergic stimulation resulting in changed feeding responses (Leibowitz, 1970).

APPENDIX 5 PRELIMINARY INVESTIGATION OF THE DISTRIBUTION OF AGENT IN SPLEEN CELLS THREE WEEKS AFTER AN I.C. INJECTION OF 79A IN VL MICE

The possibility that very specific cell populations in the lymphoreticular system could be required for scrapie pathogenesis (see SECTIONS 3 and 4) prompted the following preliminary attempt to detect a difference in scrapie titre between the glass-adhering and the non-adhering cells from the spleens of VL mice, three weeks after an i.c. injection of 79A agent.

Material and Methods. Three weeks after an i.c. injection of standard 79A supernate, the spleens were removed aseptically from two VL females. By this time (assuming a similar time course to that of the ME7 agent in C57 mice; see Dickinson & Fraser, 1969) there should have been a considerable amount of replication of the agent over and above the initial accumulation of inoculum. Small pieces of the two spleens were immediately frozen at -20°C for subsequent estimation of titre. The rest of the two spleens was gently teased apart in cold Hank's balanced salt solution (HBSS) and then pressed through sterile wire gauze to separate the cells. The capsule was discarded.

Separation of macrophage-enriched and lymphocyte-enriched populations of cells was achieved by a simple sequential glass-adherence technique. For this the cells were sown (about 10^7 cells/ml.) in 15 mls. HBSS in flat-sided culture bottles and incubated at 37°C for one hour initially. Then the supernatant, containing a lymphocyte-enriched population was decanted into a fresh culture bottle. This process was repeated three more times: after a further one-hour and

two half-hour periods. Meanwhile the glass-adhering, i.e. macrophage enriched populations, were detached from the bottles with a rubber policeman and stored cold to discourage further adherence. Suspensions of both cell types were lightly centrifuged at 200g for 10 minutes and then resuspended in 1 ml. of isotonic saline. After counting, the dilutions were adjusted to give suspensions of 25×10^6 cells/ml. These were frozen-thawed several times to kill and disintegrate the cells which were then thoroughly homogenised with a teflon-in-glass homogeniser. Centrifugation at 500 g for 10 mins. removed the larger pieces of cell debris.

A standard inoculum was prepared from the whole spleen fragments which had been removed and frozen earlier. Inocula were also prepared from both the macrophage-enriched and lymphocyte-enriched supernates (containing 5×10^5 cells/0.02 ml. dose) and further 10 and 100 times dilutions of these (containing agent from 5×10^4 and 5×10^3 cells per dose respectively).

Groups of 6 or 7 VL mice were injected i.c. with 0.02 ml. of either the standard spleen supernate (controls) or one of the three dilutions of either the macrophage-rich or lymphocyte-rich supernates, or of an equal mixture of these supernates. Incubation periods and lesion profiles were obtained as described in SECTION 2.

Results The incubation periods are shown in Table A5.1. The incubation period for the whole spleen supernate i.c. was 160 ± 4 days. There were no obvious differences in the lesion profiles of the various groups.

Table A5.1

Type of cell-donor	Incubation periods		
	5×10^5 cells	5×10^4 cells	5×10^3 cells
'macrophages'	175 ± 3	201 ± 7	207 ± 6*
'lymphocytes'	174 ± 4	194 ± 4	210 ± 4*
macro-lymphocyte mixture	179 ± 8	-	-

* one survivor

Discussion These results show a much higher titre than was expected but give a strong indication that the infectious unit/cell ratio was much the same in both populations.

On the basis of three titrations performed in these laboratories using ME7 brain in C57, RIII and VL mice it is possible to estimate that the initial supernates from 5×10^5 cells contained $10^{3.5}$ or about 3000 infectious units. This gives a cell/infectious unit ratio in the order of 200/1 in both the macrophage-enriched and lymphocyte-enriched materials. There is no way of knowing if this represents a high infection of a few cells or a low infection of many. Had there been a clear bias in favour of either of the populations these results could have supported some tentative conclusions. As they are, however, they are susceptible of several kinds of interpretation including the following:

- equal numbers of living infected cells in the macrophage population and dead infected cells in the 'lymphocyte' population;

- two (or more) kinds of infected cells nearly equally distributed amongst glass-adhering populations and non-adhering populations;

- large numbers of leucocytes of several types bearing agent on their surfaces. This could be an accumulation of the original supernate with or without the addition of replicated agent, or even an artifactual accumulation of agent shed by disruption of a sub-population of very fragile cells in which the agent had been replicating.

Since this experiment was performed, a more elaborate attempt at identifying scrapie-infected sub-populations of spleen cells has been published by Lavelle et. al. (1972). Their results are not comparable with those described here since they examined the spleens of terminal animals. However, it is now clear that the results from this type of experiment are bound to remain ambiguous until much more is known about the dynamics of agent-leucocyte interactions. These are being investigated in these laboratories using <u>in vitro</u> systems

Acknowledgements

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Work of this kind is enormously dependent upon the daily offices of numerous assistants whose patient, scrupulously accurate and often tedious routine work is its very foundation and without which many ideas would have to remain untested. In particular I would like to thank Miss V.M.H. Meikle for her efficient and helpful overseeing of the mouse colony; Mrs. S. Scott who bore the brunt of the routine weighing of mice, their food and water, once the system had been evolved; Miss L. Barnetson who had charge of most of my experimental animals and somehow managed to read my handwriting; Mr. D.M. Taylor who tried successfully, to keep my animals free of other pathogens without upsetting their behaviour too much; Mrs. P. Robertson and Miss M. Bruce who together with Dr. Fraser performed all the routine histological examinations and scoring; lastly, Miss M. Adam, for her endurance in drafting and redrafting this typescript, always with great accuracy and care. To these and others I have not mentioned I am very grateful, even if only for lending an ear at a time when an idea needed to be clarified or abandoned: it has been a privilege to work with such a team.

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Personal Communications

R.I. Carp, New York State Institute for Research in Mental Retardation, Staten Island, New York.

A.G. Dickinson, A.R.C. Animal Breeding Research Organisation, West Mains Road, Edinburgh EH9 3JQ.

H. Fraser, Moredun Research Institute, Edinburgh EH17 7JH.

T. Oliver, Transplantation Immunology Unit, London Hospital Medical College, Turner Street, London E.1.

G.G. Shaw, Department of Pharmacy, University of Nottingham, Nottingham, NG7 2RD.

P.C. Licursi New York State Institute for Research in Mental Retardation, Staten Island, New York.

D.M. Taylor A.R.C. Animal Breeding Research Organisation, West Mains Road, Edinburgh EH9 3JQ.